EVALUATION OF SERUM BIOMARKERS OF COLLAGEN TURNOVER IN CATS WITH HYPERTROPHIC CARDIOMYOPATHY

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfillment of the Requirements

for the Degree of Master of Science

Department of Companion Animals

Faculty of Veterinary Medicine

University of Prince Edward Island

Erin L. Trageser
Charlottetown, Prince Edward Island
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Examiners:

Etienne Côté, co-supervisor _____

Sherri Ihle, co-supervisor _____

Shelley Burton, committee member _____

Hans Gelens, committee member _____

Shannon Martinson, committee member _____

Tarek Saleh, committee member _____

Shannon Martinson, committee member _____

Sandra McConkey, cxternal examiner _____

Andrea Matthews, examination committee_____

Peter Foley, examination committee chair _____

Date _____

ABSTRACT

BACKGROUND Myocardial fibrosis is a hallmark of hypertrophic cardiomyopathy (HCM). Human patients with HCM have high serum concentrations of biomarkers of collagen synthesis, indicating a profibrotic state. This abnormality exists in sarcomere mutation carriers both with and without overt left ventricular hypertrophy, suggesting its potential use in early identification of HCM. Whether serum levels of biomarkers of collagen synthesis are increased in cats with HCM is unknown.

HYPOTHESIS: We hypothesized that serum biomarkers of collagen turnover could be measured in cats using commercially available enzyme-linked immunosorbent assays (ELISAs), that cats with HCM would have higher serum concentrations of a collagen synthesis biomarker than normal cats, and that a collagen degradation biomarker would not differ between cats with HCM and normal cats.

METHODS Cats presenting to a university veterinary cardiology referral service were prospectively evaluated and enrolled in the study when they had an echocardiographic diagnosis of HCM or no structural heart disease. Cats with a high serum thyroid hormone (T4) concentration, high systolic arterial blood pressure, recent or ongoing diuretic administration, echocardiographic diagnosis of cardiomyopathies other than HCM or of other congenital or acquired lesions, and/or overt signs of hypovolemia, were excluded. The diagnosis of HCM was based on echocardiographic measurements of the interventricular septum (IVS) and/or left ventricular free wall (LVFW) >5.5 mm in thickness. Cats with IVS and LVFW <5.5 mm and no other echocardiographic abnormalities were considered normal. Serum concentrations of C-terminal propeptide of type I procollagen (PICP, marker of collagen synthesis) were attempted to be evaluated using 2 commercially available ELISAs. One was previously validated for use in humans (Microvue™ EIA kit; Quidel®) and one in rats [ELISA kit for procollagen type I C-terminal propeptide (PICP); Uscn Life Science Inc.] A commercially available ELISA previously validated for use in cats (Serum CrossLaps® ELISA; Nordic Bioscience Diagnostics*)* was used to quantify serum concentrations of C-terminal telopeptide of type I collagen (CTx) as a marker of collagen degradation. We compared serum [CTx] of cats with HCM and controls.

RESULTS Forty-seven cats qualified for and participated in the study: 28 with an echocardiographic diagnosis of HCM and 19 normal controls. Serum samples from 12 additional cats with unknown echocardiographic statuses were also used in assay evaluation. In 22 experiments (8 for PICP using 1 ELISA, 10 for PICP using a second ELISA, and 4 for CTx) conducted on a total of 114 blood samples from 59 cats and 4 rats, stability, precision and linearity testing failed to provide reliable results for quantifying serum [PICP] using either ELISA. There was no difference in serum [CTx] between cats with HCM and normal controls (HCM: mean 0.248 ng/ml; controls: mean 0.253 ng/ml; p=0.4).

CONCLUSIONS The 2 ELISAs evaluated are not valid for quantifying serum [PICP] in the cat; therefore, we are unable to conclude whether or not serum biomarkers reflect increased collagen synthesis in the pathophysiology of HCM in cats. There was no evidence of enhanced collagen degradation in cats with HCM compared to normal controls.

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LIST OF ABBREVIATIONS

β-MHC: beta-myosin heavy chain
BNP: B-type natriuretic peptide
CAT: Cat Action Team
CICP (also PICP, PIP): carboxyterminal propeptide of type I procollagen
cMRI: cardiac magnetic resonance imaging
CTX (also ICTP): carboxyterminal telopeptide of type I collagen
CV: coefficient of variance
ECM: extracellular matrix
ELISA: enzyme-linked immunosorbent assay
ELISAs: enzyme-linked immunosorbent assays
FS: fractional shortening
FORL: feline osteoclastic resorptive lesions
HCM: hypertrophic cardiomyopathy
ICTP (also CTx): carboxyterminal telopeptide of type I collagen
IVS: interventricular septum
LA: left atrium
LA:Ao: left atrial to aortic ratio
LAX: long axis
LGE: late gadolinium enhancement
LV: left ventricle
LVID: left ventricular internal diameter
LVFW: left ventricular free wall
LVH: left ventricular hypertrophy
LVOT: left ventricular outflow tract
MBPC: myosin-binding protein C
MMP: matrix metalloproteinase
MRI: magnetic resonance imaging
NT-proBNP: amino(N)-terminal of B-type natriuretic propeptide
OA: osteoarthritis
OD: optical density
PICP (also CICP, PIP): carboxyterminal propeptide of type I procollagen
PIIINP: aminoterminal propeptide of type III procollagen
PIINP: aminoterminal propeptide of type II procollagen
PINP: aminoterminal propeptide of type I procollagen
PIP (also PICP, CICP): carboxyterminal propeptide of type I procollagen
RVOT: right ventricular outflow tract
SAM: systolic anterior motion (as in SAM of the mitral valve)
SAX: short axis
TMB: tetramethylbenzidine

I. GENERAL INTRODUCTION

1.1 Overview and purpose of work

Hypertrophic cardiomyopathy (HCM) is a highly prevalent disease in the domestic cat population, and feline HCM is a well recognized animal model for HCM in people.[1-5] Initiated by genetic mutations in proteins of the cardiac sarcomere, the disease produces characteristic histologic changes including myocyte hypertrophy and disarray, coronary microvascular remodeling, regional myocardial ischemia, and remodeling of the extracellular matrix (ECM).[2,5-10] Within the ECM, signaling pathways stimulate excessive fibroblast secretion and deposition of collagen with subsequent interstitial fibrosis.[11-12] Associations between myocardial fibrosis and sudden cardiac death, ventricular arrhythmias, and congestive heart failure have been documented in human HCM patients.[11,13,14]

Serum biomarkers of collagen turnover have been investigated in people with disorders resulting in myocardial fibrosis. Specifically, serum concentrations of the carboxyterminal of the propeptide type I procollagen (PICP, CICP, PIP), the aminoterminal of the propeptide type I procollagen (PINP), and the carboxyterminal telopeptide of type I collagen (ICTP, CTx) have been shown to reflect synthesis (PICP, PINP) and degradation (ICTP, CTx) of type I collagen.[13,15,16] Changes in serum biomarker concentrations indicative of disequilibrium in collagen synthesis and degradation have been documented in people with hypertensive heart disease, acute myocardial infarction, and HCM.[17-23]

These findings culminated in a recent study that identified increased serum concentrations of PICP in asymptomatic human patients with HCM compared to normal

controls. [24] This finding was considered consistent with a profibrotic state in patients who demonstrated the disease phenotype as well as in first-degree relatives of those patients who were genotypically affected but phenotypically normal.[24] Investigators concluded that the profibrotic state in patients with HCM may be identified using these serum biomarkers prior to clinical recognition of left ventricular hypertrophy (LVH). Given similarities in the pathogenesis and lesions of HCM between humans and cats, we proposed to investigate whether an association exists between HCM and serum concentrations of collagen biomarkers in cats. We hypothesized that serum concentrations of PICP could be measured in cats using a commercially available enzyme linked immunosorbent assay (ELISA) and that cats with HCM would have higher serum concentrations of PICP than normal cats. We further hypothesized that there would be no difference in the circulating serum concentrations of CTx between cats with HCM and normal cats and that the ratio of these biomarkers (PICP:CTx) would indicate a profibrotic state in cats with HCM. Ultimately, the value of this study would enhance the understanding of myocardial fibrosis in feline HCM and in early recognition of the disease in cats.

II. HYPERTROPHIC CARDIOMYOPATHY

2.1 Introduction

Hypertrophic cardiomyopathy (HCM) is a disease characterized by hypertrophy of the left ventricular (LV) myocardium in the absence of primary causes of compensatory concentric hypertrophy such as aortic stenosis, systemic hypertension, and hyperthyroidism.[25-28] The Maine Coon cat serves as a spontaneously occurring model for familial HCM in humans.[3,4,27] This disease is reported to affect as many as 1/500 human adults in the general population, making it the most common genetic cardiomyopathy, and it is the leading cause of sudden death in young male athletes.[29-32] Comparatively, the prevalence of HCM in cats is suggested to be much higher, with one recent study reporting a prevalence as high as 15.5% in a population of asymptomatic cats.[4,33]

2.2 Genetic basis and pathogenesis

Primary genetic mutations in proteins of the sarcomere are identified in approximately 2/3 of humans with HCM, and molecular studies suggest an autosomal dominant mode of inheritance.[34-36] Over 1,400 pathologic mutations affecting at least 11 different proteins have been identified in humans, with the most commonly affected proteins being beta-myosin heavy chain (β-MHC) and myosin-binding protein C (MBPC).[27,37,38] In comparison, only two mutations, both affecting genes that code for MBPC, have been identified in association with HCM in cats; these are reported in Maine

Coon and Ragdoll breeds.[39,40] These mutations interrupt the normal structure and function of the sarcomere and ultimately result in decreased force production.[41] As a result, stress-responsive growth factors, including myocyte enhancer factor, transforming growth factor, and connective tissue growth factor, are produced in greater concentrations and cause myocyte hypertrophy and fibroblast proliferation.[18,42] Myocardial fibrosis is hypothesized to arise from both excessive fibroblast stimulation (with excessive secretion of collagen) and from hypoxia (when hypertrophy exceeds the anatomic limits of normal coronary perfusion).[43-45]

In addition to interstitial and replacement fibrosis, other hallmark histologic features of HCM include myocyte hypertrophy and disarray and coronary arteriosclerosis.[6-9] These changes have been variably described in other unrelated anatomical and physiological circumstances, suggesting that no single clinical or histologic feature of HCM is pathognomonic.[42,45] Proposed mechanisms for myocyte disarray include increased wall stress and prolonged isometric contraction, alterations in the normal direction of myocardial forces, and interference with the normal parallel alignment of myocardial fibers.[9]

These pathophysiologic characteristics produce at least three manifestations of clinical disease: diastolic dysfunction, LV outflow tract obstruction, and myocardial ischemia.[27] Diastolic dysfunction results from the cumulative effects of disorganized cellular architecture and fibrosis on the passive elastic properties of the LV.[46] Gwathmey et al. also showed that impaired calcium regulation leads to increased intracellular calcium concentrations and thus impaired active relaxation.[47] Diastole is physiologically divided into 4 phases: isovolumic relaxation, rapid ventricular filling, diastasis, and atrial

systole. In HCM, diastolic dysfunction is characterized by a decreased rate and volume of LV filling during isovolumic relaxation. This prolongs rapid filling time and promotes a compensatory increase in atrial systolic function in order to maintain normal ventricular filling volume.[48] Impaired ventricular relaxation and enhanced atrial systolic function result in elevated left atrial pressure, a predisposing factor for left-sided congestive heart failure. [48,49]

Left ventricular outflow tract (LVOT) obstruction results from either hypertrophy at the base of the interventricular septum (IVS) and/or from systolic anterior motion (SAM) of the mitral valve, both of which create a physical impediment to the flow of blood out the aorta during systole. Systolic anterior motion of the mitral valve was historically proposed to arise from the Venturi effect. This is a narrowing of the LVOT which creates high velocity blood flow that tows the anterior leaflet of the valve into the outflow tract, creating additional obstruction.[50] This explanation has recently lost favor. Instead, it is currently accepted that hypertrophy of the papillary muscles distorts chordae tendinae attachments to the leaflet, displacing it into the LVOT during systolic ejection.[51] The effect of outflow tract obstruction is increased intraventricular systolic pressure, causing increased myocardial wall stress and myocardial oxygen demand. Increased wall stress is associated with progressive and compensatory concentric hypertrophy to provide the necessary force to maintain normal stroke volume despite LVOT obstruction. Mitral valve SAM imposes the additional detriment of disrupting the integrity of the mitral valve apparatus during systolic ejection, creating a conduit for mitral regurgitant flow. Mitral regurgitation contributes additional volume to the LA, further increasing LA pressure.

Myocardial ischemia results from discordance between myocyte hypertrophy and anatomical perfusion limits of the coronary vasculature, inadequate capillary density, and small-vessel disease of the intramural coronary arteries.[3,7,10] Ischemic myocytes are further prone to calcium mishandling, impaired relaxation, and cell death.[52] When ischemic myocytes die, cellular repair mechanisms are stimulated to create replacement fibrosis to maintain structural integrity.[42] Myocardial fibrosis imposes an additional viscoelastic burden that further impedes diastolic filling.[46] In its entirety, the pathogenesis of HCM can be summarized as sarcomeric genetic mutations that alter myocardial structure, composition, and function, resulting in diastolic dysfunction and elevated intracardiac pressures.

2.3 Clinical recognition of disease

In both people and cats, clinical manifestations of HCM cover a broad range from complete absence of clinical signs to incidentally ausculted heart murmurs to congestive heart failure and sudden cardiac death. Asymptomatic affected people are commonly identified through clinical and genetic screening prompted by diagnosis of a symptomatic family member.[35,37] Comparatively, the paucity of known disease causing mutations in cats, evidence of incomplete penetrance of the mutated genes encoding MBPC, and the high prevalence of asymptomatic carriers limits the diagnostic yield of genetic screening in this species.[33,53-55] Genetic testing in cats is largely reserved for research or for Maine Coon and Ragdoll breeding programs. Incidentally identified heart murmurs are a common reason for evaluation resulting in a diagnosis of HCM in asymptomatic cats.[56,57]

However, one study reported a positive predictive value of only 33%, making heart murmur identification a test of relatively low sensitivity for diagnosing HCM.[4]

Definitive diagnosis depends on the identification of myocardial hypertrophy in the absence of causative systemic hypertension, hyperthyroidism, aortic stenosis or other primary lesions. In people, hypertrophy is clinically demonstrable using either echocardiography or cardiac magnetic resonance imaging (cMRI), while echocardiographic diagnosis is most commonly used in cats. The advantages of cMRI over echocardiography in people include thorough evaluation of cardiac function, accurate calculation of LV mass, assessment of regional wall abnormalities, and contrast enhancement of myocardial fibrosis.[27,28,58] While cMRI is accurate for measuring LV mass in cats with HCM, a study of 26 affected Maine Coon cats and 10 normal controls revealed contrast enhancement in only one affected cat, suggesting low utility of cMRI for identifying myocardial fibrosis in cats.[59] Thus, echocardiography is the current diagnostic test of choice for feline HCM. Specific diagnostic criteria have been established for the thickness of the LV free wall (LVFW) and/or IVS, left atrial size, the presence or absence of SAM of the mitral valve, and color and spectral Doppler criteria that support LVOT obstruction.[2,5,33,60]

1.4 Clinical utility of serum biomarkers: natriuretic peptides

Based on the need for relatively expensive and limited equipment and expertise necessary for echocardiographic evaluation, there is recent interest in the role of serum biomarkers in the diagnosis of HCM. Cardiac biomarkers are circulating substances that

reflect myocardial structure or function, and concentrations above normal reference intervals may indicate the presence of cardiac disease. Serum biomarker testing is attractive due to its affordability, relatively wide availability, and potential utility in screening, prognosticating, and monitoring therapeutic management of heart disease in people as well as in cats.

One of the most prominent recent biomarkers under investigation is a member of the natriuretic peptide family of hormones. Natriuretic peptides are produced in the human and feline myocardium under circumstances of myocardial stretch or stress.[61] A precursor molecule of B-type natriuretic peptide (BNP) called preproBNP originates from both atrial and ventricular myocytes, more so ventricular in pathologic states.[61,62] After secretion of preproBNP, two flagging domains, the amino (N) and carboxy (C) terminals, are cleaved and excreted renally. The BNP hormone is then activated to serve in natriuresis and diuresis.[61] The N-terminal domain is cleaved in 1:1 proportion with the active hormone, and its half-life in human and feline serum prior to renal excretion is longer than BNP.[61,62] Therefore, measurement of NT-proBNP has become a diagnostically useful biomarker in the identification of various myocardial diseases in humans and animals.[61-65] There is conflicting evidence regarding the reliability of using serum concentrations of NT-proBNP to diagnose asymptomatic HCM in cats.[66-68] Early investigation limited to a research colony of Maine Coon cats showed no statistically significant differences in serum concentrations of NT-proBNP between cats with asymptomatic HCM versus normal controls.[66] Subsequent studies that enrolled greater numbers of cats of various breeds showed that those with HCM had significantly higher serum concentrations of NT-proBNP than those without HCM and that serum

concentrations were associated with the severity of disease.[67,68] Increased activity is currently devoted to the study of veterinary cardiac biomarkers. A reliable serum diagnostic test would ideally enable early diagnosis of HCM in cats, leading to improved owner education, enhanced monitoring, and appropriate lifestyle modification with or without medical intervention. Likewise, an appropriately specific biomarker assay would afford reassurance to cat owners and veterinarians when results indicate the absence of disease.

Circulating concentrations of the extension peptides of related A-type natriuretic peptide (NT-proANP and CT-proANP) have similarly been shown to be higher in cats with HCM compared to normal controls.[69,70] Clinical use of this biomarker is limited, however, due to lack of a feline-specific assay. The C-, D-, and V-type natriuretic peptides have not been investigated in HCM since C-type natriuretic peptide is more specific to the vasculature than the myocardium, and D- and V-types have been described only in non-feline species.[71]

2.5 Myocardial fibrosis and collagen

As previously described in humans with HCM, serum biomarkers of collagen turnover offer promising surrogate measurements of myocardial fibrosis in cats with HCM. The interstitial and remodeling fibrosis described in the pathogenesis of HCM arises from dysregulation between collagen synthesis and degradation within the extracellular matrix (ECM) of the myocardium.[1,7,8,11,12] In health, the function of the ECM is to provide a scaffold for the structural support of adjacent myocytes and vessels

and to translate the force generated by individual myocytes into organized ventricular contraction; it also accounts for passive stiffness during diastolic filling of the ventricles.[72] The ECM is predominantly composed of fibrillar collagen types I and III (and less abundantly types IV, V and VI), fibronectin, laminin, elastin, fibrillin, proteoglycans and glycoproteins.[42,73] The source of collagen is cardiac fibroblasts, which account for the largest cell population in the myocardium.[73] Normal turnover of the collagenous ECM occurs regularly with protein secretion by fibroblasts and degradation by matrix metalloproteinases (MMPs) which are activated by extracellular serine proteases.[74]

Cardiomyocyte injury or sustained pressure or volume overload induces increased production of growth factors such as platelet-derived growth factor, basic fibroblast growth factor, and transforming growth factor β.[74-76] These growth factors trigger fibroblasts to increase secretion of ECM proteins, particularly collagen.[75,76] In excess, the tensile strength of collagen can impose abnormally increased stiffness on the myocardium in both systole and diastole.[77] Accumulation of collagen occurs in two morphologically distinct patterns based on the alignment of thick and thin collagen fibers to one another and to cardiac muscle.[78] These patterns can represent a reparative process in response to myocardial infarction and necrosis, or a reactive process in response to inflammation without necrosis.[77-79] Generally, reparative fibrosis occurs primarily in the interstitial space while reactive fibrosis occurs primarily perivascularly; both are equally identified in normal cardiac remodeling.[72,74] Fibrosis identified in association with cardiac lesions, including HCM, occurs as a reparative process with predominantly interstitial fibrosis and relatively less perivascular fibrosis.[78] The degree of each type of

fibrosis depends on the nature and severity of insult.[79] After myocardial injury, the remodeling process is initiated by collagen fiber degradation, interstitial edema, and increased secretion of collagen, with fibrous connective tissue occupying as much as 30% of the myocardium.[79,80] Substantial deposition of fibrotic tissue alters the mechanical properties of myocardial infrastructure, causing increased stiffness, impaired diastolic filling, increased energy requirements and decreased oxygen diffusion, resulting in a hypoxic substrate for arrhythmogenesis.[79-81] Thus, although fibrosis is initiated as a compensatory repair mechanism in myocardial injury, its effects further propagate myocardial dysfunction.

2.6 Myocardial fibrosis in heart disease

Increased myocardial fibrosis has been reported in association with a variety of cardiovascular diseases in humans. The most common of these are hypertensive heart disease, acute myocardial infarction, heart failure with preserved ejection fraction, dilated cardiomyopathy, and HCM.[13,17-21,78] In these diseases, myocardial fibrosis can be identified on imaging modalities such as 2-dimensional echocardiography or cMRI. The functional effect of myocardial fibrosis is demonstrated as decreased longitudinal strain on strain imaging echocardiography, with the greatest decrease in strain correlating to the regions of most extensive fibrosis.[82] Combined imaging and post-mortem studies in people and cats with HCM provides evidence that the presence, amount, and patterns of fibrosis vary and that fibrosis is not correlated with the location or extent of hypertrophy.[83-85]

Previous reports indicate that up to 80% of human patients with HCM have myocardial fibrosis as identified by late gadolinium enhancement (LGE) on cMRI.[86,87] The presence and extent of fibrosis identified on cMRI has been associated with progressive ventricular dilation, ventricular arrhythmias, and sudden cardiac death in people with HCM.[88,89] In addition, cMRI findings have incremental value for risk stratification in the therapeutic management of HCM patients.[90] As noted previously, cMRI in cats with HCM has not mirrored these findings, and its use is currently investigational only.

Endomyocardial biopsy (EMB) has been used as a diagnostic modality for identifying the characteristic histologic abnormalities of HCM. Studies comparing EMB to echocardiographic and post-mortem findings show that the intrinsic myopathic process is not limited to hypertrophied regions nor is it always panmyocardial[91,92] Therefore, EMB may not be wholly representative of the myocardium, and samples from affected patients may lack remarkable pathologic findings.[91,92] In addition, EMB in cats would require general anesthesia and specialized costly equipment, making its use impractical.

2.7 Biomarkers of collagen turnover

Serum biomarker measurement offers an additional diagnostic modality for assessing myocardial fibrosis. Both fibrillar collagen types I and III comprise the cardiac ECM.[93,94] Both types are synthesized as pro-collagens that undergo endoproteinase cleavage of their propeptide extension domains within the extracellular space before the mature collagen molecule cross-links and assembles into collagen fibers.[93,94] The

extension peptides are the amino (N) terminal and carboxy (C) terminal ends, and they are released in a stoichiometric ratio to the active collagen molecule. [16] Both extension peptides are found in the vascular space, where they can be measured in serum.[16,94] The C-terminal propeptide is stabilized by interchain disulfide bonds which the N-terminal propeptide lacks; this makes its measurement superior to measurement of the N-terminal when quantifying type I collagen synthesis.[16,95] Specifically, the C-terminal of type I collagen (PICP) is considered an excellent marker for collagen synthesis and is used for quantifying type I collagen synthesis in the investigation of various human diseases.[16,96-98] Both radioimmunoassays and sandwich enzyme linked immunosorbent assays (ELISAs) have been developed for quantifying PICP in serum and plasma from both humans and rats.[15,16,98-101]

Collagen fibers are degraded when bone demineralizes during normal physiologic turnover or pathologic destruction. During collagen fiber degradation, similar N- and C-terminal extension peptides, now called telopeptides, of the collagen molecules are cleaved by MMPs. The telopeptides consist of non-helical sequences of collagen at each end of the molecule and, upon release during collagen disassembly, can be measured in the serum as surrogate markers of collagen degradation. [102] The C-terminal end (ICTP), which includes the non-helical telopeptide and a terminal helical segment, and the C-terminal end without the helical segment (CTx), are measurable using different commercially available ELISAs. [102,103] These assays have been utilized to investigate bone metabolism in diseases such as osteoporosis, hyperparathyroidism, and neoplasia.[102,103]

Figure 1 illustrates a simplified depiction of the interaction between these

measurable domains of type I collagen. Trimerization of the collagen molecule after N- and C-terminal propeptide cleavage is excluded from the figure for the purpose of exclusively demonstrating origin of the biomarkers measured by routine assays. Collectively, assays that measure serum concentrations of PICP and CTx offer a means of non-invasive testing which provide insight into collagen activity.

Since collagen is not exclusive to the myocardium and is more abundant in bone, original investigation of biomarkers of collagen turnover occurred in human patients with bone disease. These diseases included osteoporosis and osteogenesis imperfecta, and affected individuals were shown to have lower than normal serum concentrations of PICP and/or higher than normal serum concentration of ICTP or CTx.[104,106] Fibrosis of other organ systems was also shown to result in increased serum concentrations of PICP.[107]

Measurement of collagen biomarkers in humans has provided insight into cardiac ECM remodeling in various heart diseases. In one study, hypertensive patients had increased serum PICP concentrations compared to normal controls, and PICP concentrations correlated with collagen volume fraction in myocardial tissue samples.[14] Notably, hypertensive heart disease diffusely affects the LV, in contrast to HCM, where histologic changes occur multifocally and are not uniformly represented on EMB. Patients with hypertensive heart disease also had lower serum ICTP concentrations than normal control patients in one study.[108] The investigators suggested that this reflected myocardial collagen deposition without equal degradation, the net result being fibrosis.[108] Another study showed a correlation between collagen synthesis (as represented by elevated serum concentration of PINP) and echocardiographic parameters of diastolic dysfunction in hypertensive patients.[109]

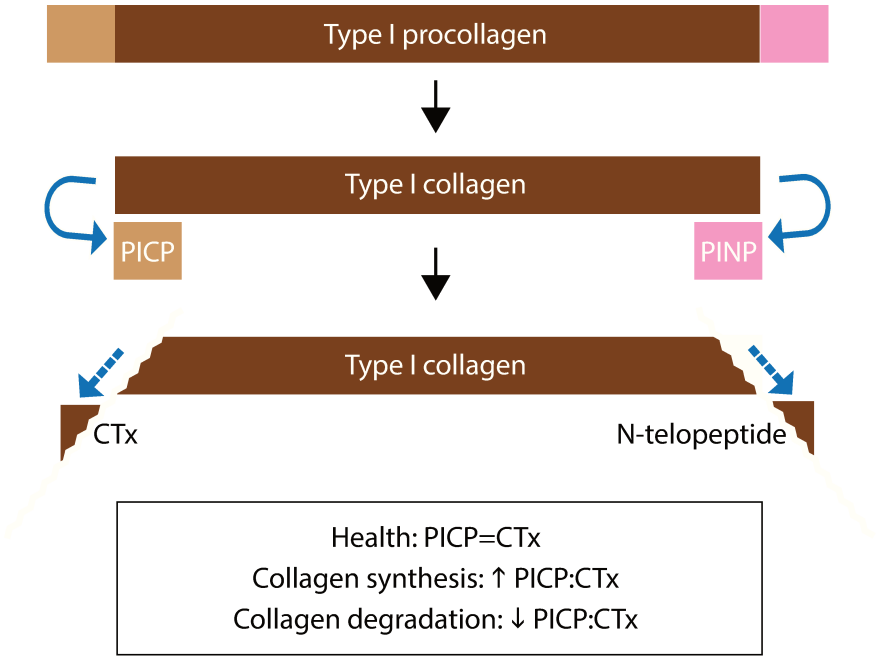

Figure 1. Extension domains of normal collagen turnover. Carboxyterminal propeptide of type I procollagen (PICP) and carboxyterminal telopeptide of type I collagen (CTx)

In addition to systemic hypertension, the high incidence of myocardial infarction in people has provided ample opportunity to investigate serum collagen biomarkers in reparative myocardial fibrosis. Increased serum concentrations of the N-terminal domains of types I and III procollagens (PICP and PIIINP) and decreased ICTP or CTx concentrations are associated with the extent of tissue ischemia and poorer survival times after acute myocardial infarction.[110-112] Diseases like systemic hypertension, coronary artery disease, and myocardial infarction are commonly implicated in the development of myocardial fibrosis, which is associated with poor clinical outcomes.[112-119]

2.8 Collagen biomarkers and HCM

Enhanced cardiac collagen content and collagen turnover have been demonstrated in people with HCM compared to normal controls.[11,18,21,120] Ho et al. recently demonstrated elevated serum concentrations of PICP in humans with genetic mutations causing HCM, regardless of whether myocardial hypertrophy was demonstrable using echocardiography or cMRI.[24] In addition, circulating serum concentrations of ICTP did not differ between mutation carriers (with and without hypertrophy) and normal controls.[24] The resultant greater ratio of PICP:ICTP in clinical HCM patients indicated a trend toward ongoing collagen synthesis and deposition without a reciprocal degree of degradation. Normal control subjects, consisting of first degree family members without the mutation, did not have increased serum concentrations of PICP or ICTP. Investigators surmised that serum biomarkers demonstrated altered collagen turnover prior to clinical recognition of myocardial fibrosis and, as such, postulated that

myocardial fibrosis may be both a contributor to HCM pathogenesis as well as a result of remodeling. They concluded that measurement of serum collagen biomarkers may supplement the early clinical identification of affected individuals.[24] This work suggests that serum PICP concentration may be a sensitive biomarker for differentiating normal hearts from hearts with HCM at its earliest identifiable stage. Furthermore, the balance between PICP and ICTP concentrations may elucidate the pathophysiologic mechanism of fibrosis associated with HCM. Given similarities in the genetic basis, pathogenesis, and clinical manifestation of HCM between humans and cats, we designed a study to investigate serum biomarkers of collagen turnover in cats with HCM.

2.9 Collagen biomarkers in animals

In addition to experimental models of human disease, collagen biomarkers have been investigated in animals under a number of different circumstances with particular emphasis on orthopedic diseases.[121,122]

In a study of experimentally-induced canine osteoarthritis (OA), serum concentrations of the C-terminal propeptide of type II collagen (PIICP) were not significantly different from baseline at either 3 or 12 weeks post-surgical resection of the cranial cruciate ligament.[123] Other studies using the same model for stifle OA in dogs have documented alterations in PIICP concentrations consistent with the destruction of articular cartilage.[124,125] In horses with experimentally induced OA, serum concentrations of PIICP were elevated but were reportedly unreliable predictors of lameness.[126]

Compared with healthy dogs and dogs with various orthopedic diseases, dogs with appendicular osteosarcoma had significantly higher concentrations of both N- and C-telopeptides of type I collagen in serum and urine, consistent with bone destruction.[127]

Neoplasia in cats, particularly bone-invasive oral squamous cell carcinoma, was associated with increased serum CTx concentrations when compared to geriatric, healthy control cats.[128] Treatment of these patients with zoledronate, a bisphosphonate compound used to prevent skeletal fractures, resulted in decreased concentrations of CTx in affected cats.[128] Another showed significantly higher CTx concentrations in cats under 2 years of age compared to older normal cats.[129] This same study also found increased CTx concentrations consistent with collagen degradation in cats with feline osteoclastic resorptive lesions of the mouth.[129]

2.10 Value of the study

Investigating collagen serum biomarkers in cats with HCM and possible associations between these biomarkers and myocardial fibrosis could implicate enhanced collagen turnover as an earlier manifestation of HCM than previously thought. Since diagnosis can be complicated by the insensitivity of auscultable murmurs and the expense and limited availability of echocardiographic equipment and expertise, a reliable serum-based test could aid in the early diagnosis of HCM in asymptomatic cats. Thus, the value of this study lies in the potential for improving the current understanding of the pathogenesis of HCM in cats and for aiding early diagnosis. We hypothesized that serum concentrations of PICP could be measured in cats using a commercially available ELISA

and that cats with HCM would have higher serum concentrations of PICP than normal cats. We further hypothesized that there would be no difference in the circulating serum concentrations of CTx between cats with HCM and normal cats, and that the ratio of these biomarkers (PICP:CTx) would indicate a profibrotic state in cats with HCM.

In accordance with these hypotheses, we aimed to:

1) evaluate the use of commercially available ELISA assays for quantifying PICP in feline serum.

2) compare serum concentrations of PICP and CTx in cats with and without an echocardiographic diagnosis of HCM.

3) determine if any association exists between serum concentrations of PICP and CTx in cats with HCM and the presence or extent of myocardial fibrosis as suspected on echocardiographic evaluation or demonstrated on histology.

III. MATERIALS AND METHODS

The study protocol was approved by the Animal Care Committee (#10-058) at the Atlantic Veterinary College, University of Prince Edward Island. Informed, signed owner consent was obtained for each cat enrolled in the study.

3.1 Participants

Cats were enrolled in the study upon presentation to the cardiology service at the Atlantic Veterinary College Veterinary Teaching Hospital if they had an echocardiographic diagnosis of either HCM (as defined below) or no structural heart disease. Exclusion criteria included fractious or uncooperative behavior that prevented echocardiographic evaluation or atraumatic phlebotomy, an echocardiographic diagnosis of congenital or acquired heart disease other than HCM, and Doppler-derived systolic blood pressure >160 mmHg. Additional exclusion criteria included owner reported clinical signs or physical examination findings consistent with underlying extracardiac disease, particularly those that may serve as a source of inflammation or fibrosis. These included but were not limited to a history of or current azotemia, a positive feline leukemia virus or feline immunodeficiency virus ELISA result, or palpable skeletal lesions. Original study design allowed for inclusion of cats from three populations in an attempt to gather as many normal controls as HCM-affected cats. These three populations included: cats presented by clients of the veterinary teaching hospital; feral cats treated by the Cat Action Team (CAT), a non-profit organization that operates in feral cat trap, neuter, and release; and cats at the Prince Edward Island Humane Society

slated for humane euthanasia independent of this study. Feral cats from CAT were under presurgical general anesthesia during echocardiographic evaluation but chemical restraint was otherwise initiated during echocardiography only in cats who demonstrated substantial resistance to restraint. If sedation was deemed necessary, pharmacologic agent selection was left to the discretion of the attending clinician with a strong preference toward single low dose (2-10 micrograms/kilogram) dexmedetomidine and/or low dose (0.1-0.2 milligrams/kilogram) butorphanol delivered intravenously or intramuscularly. These agents were preferred in light of their well described cardiovascular effects.[130,131]

A power analysis was performed to estimate the desired number of enrolled cats that would satisfy a significant difference (95%) with reasonable power (80%). Sample size was calculated using data extrapolated from a model human study[24] wherein control subjects had a mean [+/- standard deviation (SD)] serum PICP concentration of 82.16 +/- 3.03 μg/liter compared to subjects with HCM mutations but no evidence of LVH who had a mean serum PICP concentration of 107.73 +/-4.65 μg /liter. The concentrations at the high end of the 95th percentile for the control group and at the low end of the 95th percentile for the mutation-positive, LVH-negative group were 88.2 μg /liter and 98.43 μg/liter, respectively, with a smallest difference of interest between the two of 10.23 μg/liter. Lehr's formula was used for estimating sample size for comparing two groups with an unpaired t-test or $\chi 2$ test:

$$\text{sample size} = 16/[\text{standard difference}]$$

where the standard difference was defined as the smallest difference of interest/standard deviation.[132] The formula produced a desired sample size of 6 cats.

Lehr's formula requires assumptions about desired power and effect under study to provide a quick estimate of necessary sample size; it assumes the probability of Type 1 and Type 2 errors to be 0.05 and 0.2, respectively.[133] Because this equation produced a sample size that was small and a greater number of samples were anticipated to be needed to evaluate assay performance, the sample size was recalculated. The new calculation used a smaller, and therefore more rigorous, difference of interest based on the differences between the 99th percentiles of the two populations in the same model human study.[24] The high end of the 99th percentile for the control group and the low end of the 99th percentile for the mutation-positive, LVH-negative group (91.23 μg/liter and 93.78 μg/liter, respectively) produced a smallest clinical difference of 2.55 μg/liter. Recalculation of Lehr's formula revealed a desired sample size of 54 cats (27 with HCM and 27 normal controls) with 80% power for detecting a difference between normal and affected cats.

3.2 Echocardiography

Echocardiography was performed on each cat using a LOGIQ 7 (GE Healthcare; Wauwatosa, WI) ultrasound machine, and standard 2-dimensional (2D), M-mode, color and spectral Doppler evaluations were performed as previously described.[134] Each echocardiogram was performed by a board-certified veterinary cardiologist or a cardiology resident under direct supervision of a board-certified veterinary cardiologist. Cats were restrained first in right lateral recumbency and then in left lateral recumbency on a padded table with a cut-out area for thoracic imaging from below. Fur was clipped from the right and left axillae as needed, and the skin was wetted with alcohol prior to

ultrasonographic evaluation. Simultaneous electrocardiography was recorded via electrodes secured to the palmar and plantar surfaces of all four paws with bandage material. In all cats, two-dimension (2D) images were recorded from the right parasternal long axis 4- and 5- chamber views, and the right parasternal short axis apical, mitral valve, and basilar views. M-mode images were recorded at the right parasternal short axis apical, mitral valve, and basilar views for measurements of LV diameters [including the IVS, LV internal diameter (LVID), free wall (LVFW), and fractional shortening (FS)], mitral valve, and the ratio of the diameters of the LA to the aortic annulus (LA:Ao), respectively. Color Doppler echocardiography was routinely applied to atrioventricular and semilunar valves and in cases where 2D images raised suspicion of shunting or regurgitant lesions. Spectral Doppler (pulsed-wave and/or continuous-wave) was used to measure the velocity of flow across the pulmonic valve in the right parasternal short axis basilar view and across the aortic valve in the left apical four chamber view. Right parasternal long axis 4- and 5- chamber views were also captured and recorded using an 8C probe for optimal visualization of the entire LV, including the LV apex.

Echocardiographic measurements and analysis were completed offline. Recorded measurements included the following: the diameters of the IVS, LVID, and LVFW at end-diastole and peak-systole as determined by simultaneous ECG, LA:Ao at end-diastole in both 2D and M-mode images, and the velocity of the right ventricular and LV outflow tracts. End diastolic LVFW and IVS thicknesses were additionally measured at multiple points in the 2D right parasternal long axis 4-chamber and 2D right parasternal short axis apical views as recently described by Wagner et al.[5] (Figure 2) Maximal wall

thickness was measured in each previously described region: IVS_{sax1}, IVS_{sax2}, $LVFW_{sax}$, IVS_{lax1}, IVS_{lax2}, $LVFW_{lax}$. The diagnosis of HCM was made when one or more of the segments IVSd, LVFWd, IVS_{sax1}, IVS_{sax2}, $LVFW_{sax}$, IVS_{lax1}, IVS_{lax2} or, $LVFW_{lax}$ exceeded 5.5 mm in thickness independent of a primary cardiac lesion, measurable systemic hypertension, or documented or clinically suspected hyperthyroidism. The LA:Ao was measured in 2D and M-mode images, both obtained from the right parasternal short axis basilar view, and LA enlargement was defined as an LA:Ao >1.5. Qualitative echocardiographic observations regarding myocardial hyperechogenicity, the thickness, symmetry, and/or echogenicity of papillary muscles, presence of LV false tendons, appearance of mitral valve SAM, and other structural abnormalities were recorded. Cats in the control group were assessed as having no echocardiographic evidence of structural heart disease when the LVFW and IVS were uniformly <5.5 mm in thickness, the LA:Ao <1.5, LVOT and RVOT velocities were <2.0 m/sec, and there was no 2D, M-mode, or Doppler evidence of shunts or other lesions. The presence of dynamic right ventricular outflow tract obstruction as documented on color and spectral Doppler did not, in the absence of other abnormalities, constitute evidence of structural heart disease. As this is a common cause of physiologic murmurs in healthy cats, participants with this sole finding were grouped in normal controls.[4,135,]

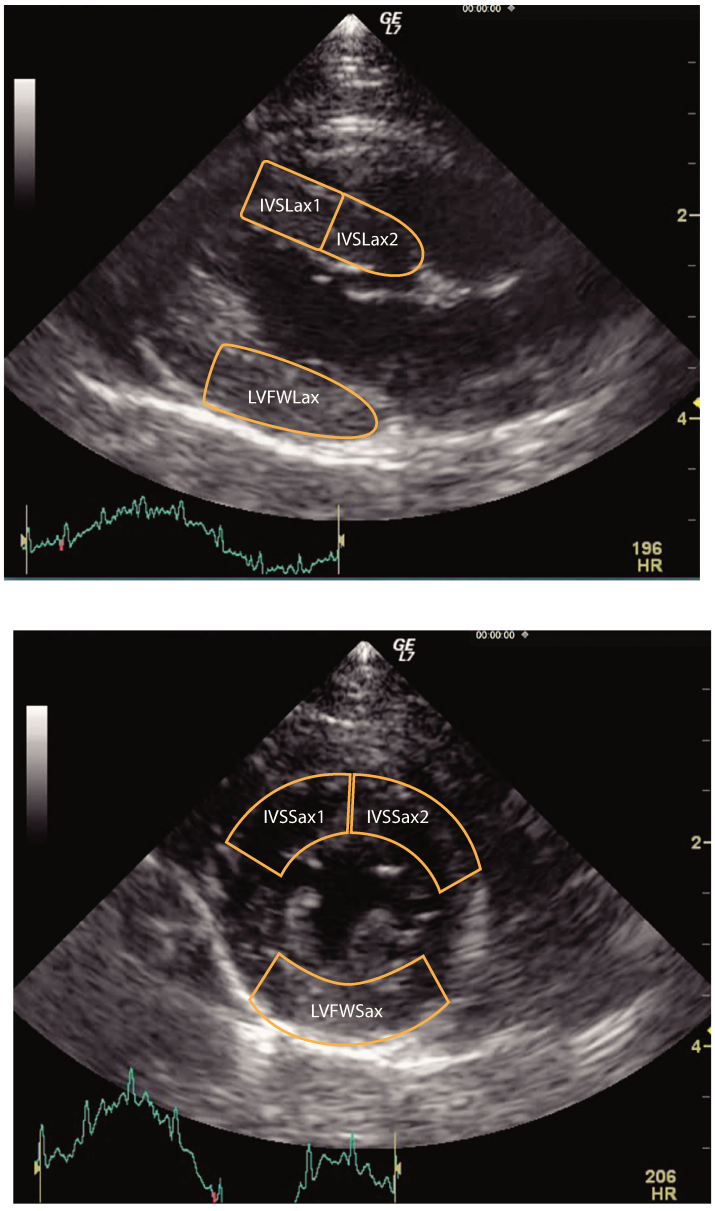

Figure 2: 2D long-axis (top) and short-axis (bottom) echocardiographic images of the left ventricle of a normal cat. IVSLax1: mid segment of the interventricular septum; IVSLax2: basal segment of the interventricular septum; LVFWLax: largest diameter of the left ventricular free wall; IVSSax1: posterior half of the interventricular septum; IVSSax2: anterior half of the interventricular septum; LVFWSax: free wall in short axis at the level of (but not including) papillary muscles

3.3 Serum biomarker evaluation

Blood samples were obtained at the time of cardiac imaging by phlebotomy of the jugular or saphenous vein using a 3 milliliter syringe and 22 or 25 gauge needle. Blood was immediately transferred to a glass tube with no indwelling anti-coagulant (Becton-Dickinson, Mississauga, ON) and allowed to clot at room temperature for up to 20 minutes. Samples were centrifuged at 300 rotations/minute (1470 G) for 15 minutes (Clinical Centrifuge Model 428, International Equipment Company, Needham Heights, MA). Following centrifugation, serum was decanted into 2 milliliter polypropylene microcentrifuge tubes in approximately 100 μL aliquots and stored at -80° Celsius prior to batch analysis. Samples that were not immediately transferrable to the -80° Celsius storage unit were stored at 4° Celsius for less than 12 hours. Aliquoting the samples at the time of initial storage was prospectively performed to reduce variation in ELISA results that could result from denaturing of proteins during multiple freeze-thaw cycles.

Serum samples were analyzed using three commercially available ELISAs, including two for PICP and one for CTx. CTx is equivalent to ICTP but without the additional helical segment; the assay used was previously validated for quantifying CTx in cat serum.[128,129]

Carboxy-terminal propeptide of procollagen type I (PICP) quantification using the Microvue™ EIA kit (Quidel® Corporation, San Diego, CA):

Serum samples of 100 μL were thawed and gently mixed. Excess shaking was

avoided to reduce the risk of shearing, denaturing, or partitioning proteins. Samples were diluted with manufacturer supplied assay buffer (comprised of nonionic detergent, stabilizer, and sodium azide [0.05%]) in dilutions of 3 or more of the following ratios: 1:4, 1:12, 1:24, 1:10, 1:20, 1:50, 1:100, and 1:200.

Manufacturer supplied standards and controls containing different concentrations of purified PICP from human fibroblast cells in a buffered solution were evaluated with each experiment. One hundred μL aliquots of each diluted sample were pipetted into wells on a 96-well microtiter plate precoated with monoclonal murine anti-PICP antibody. All standards, controls, and samples were added to the plate within 17 minutes. The plate layout was recorded for sample identification, and the plate was incubated at room temperature for 120 minutes. The wells were emptied using careful aspiration and manually washed with manufacturer supplied wash buffer diluted 1:10 with deionized water. Three hundred μL aliquots of diluted wash buffer were added to each well using a multi-channel pipette. During each wash cycle, the buffer was allowed to sit in the wells for 60 seconds. The wash was repeated two times before adding 100 μL of manufacturer supplied polyclonal rabbit anti-PICP antibody to each well. The plate was incubated at room temperature for 50 minutes, after which the wash step was repeated three more times. Then, 100 μL of manufacturer supplied lyophilized goat anti-rabbit IgG antibody conjugated to alkaline phosphatase was added to each well and reconstituted with wash buffer. The plate was allowed to incubate at room temperature for 50 minutes before repeating the wash step three additional times. P-Nitrophenyl phosphate substrate was prepared with manufacturer supplied diethanolamine and magnesium chloride buffer. After adding 100 μL of the buffered substrate solution to each well using a multi-channel

pipette, the plate was left to incubate at room temperature for 30 minutes. The reaction was stopped with the addition of 50 µL /well of manufacturer supplied NaOH stop solution. The wells were visually inspected for bubbles, blotted dry with paper towels, and the plate was read on a microplate reader at an optical density (OD) of 405 nm.

Computer quantitation software (KC4™, Bio-Tek® Instruments Inc., Winooski, VT) was used to generate a 4-parameter calibration standard curve (Figure 3) and the derived fitting equation to quantify the concentration of PICP in samples.

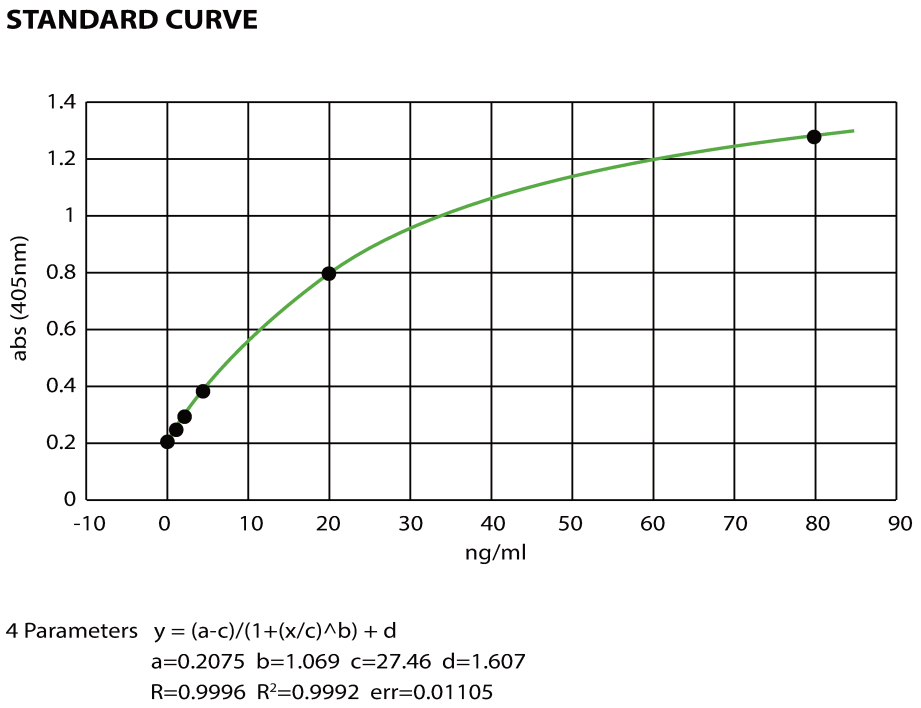

Figure 3. Sample standard curve for the MicroVue™ PICP EIA Sample standard curve and 4 parameter calibration curve-fitting equation generated by plate reader during use of the MicroVue™ PICP EIA for measuring PICP in serum

Enzyme-linked immunosorbent assay kit for procollagen type I C-terminal propeptide (PICP) (Uscn Life Science Inc., Wuhan, Hubei [P.R. China])

Serum samples of 100 µL were thawed and diluted with 0.02 molar phosphate-buffered saline (PBS) in 2 or more of the following ratios: 1:10, 1:20, 1:50, 1:100, 1:150, and 1:200. Manufacturer supplied standards and diluted samples were divided into 100 µL aliquots and added to the wells of a 96-well plate precoated with rat monoclonal antibody to PICP. The plate was covered with a plate sealer and allowed to incubate at 37°C for 120 minutes. The fluid in each well was carefully aspirated before adding 100 µL of a biotin-conjugated polyclonal antibody preparation specific for PICP.
The plate was covered with a plate sealer and allowed to incubate at 37°C for 60 minutes. The fluid in each well was carefully aspirated, and the wells were manually washed using a multi-channel pipette with 350 µL of manufacturer-supplied wash diluted 1:30 with deionized water. Because this volume nearly exceeded the capacity of the wells, a deviation in manufacturer protocol was elected, and a volume of 300 µL wash solution was substituted in all experiments after the first. The wash step was repeated four times before adding 100 µL of avidin conjugated to horseradish peroxidase. The plate was covered with a plate sealer and allowed to incubate at 37°C for 60 minutes. The wash process was repeated five times, and 90 µL of tetramethylbenzidine (TMB) substrate solution was added to each well using a multi-channel pipette. The plate was again covered with a plate sealer and allowed to incubate at 37°C for 25 minutes. The

reaction was stopped by adding 50 µL of manufacturer supplied sulfuric acid stop solution, and the plate was read on a microplate reader at an OD of 450 nm.

Computer quantitation software (KC4™, Bio-Tek® Instruments Inc., Winooski, VT) was used to construct a standard curve with the OD value of the standard on the Y-axis against the known concentration of the standard on the X-axis. The software automatically generated a line of best fit through the standard points and determined an equation via regression analysis. In diluted samples, the concentration read from the standard curve required multiplication by the dilution factor. A sample standard curve is shown in Figure 4.

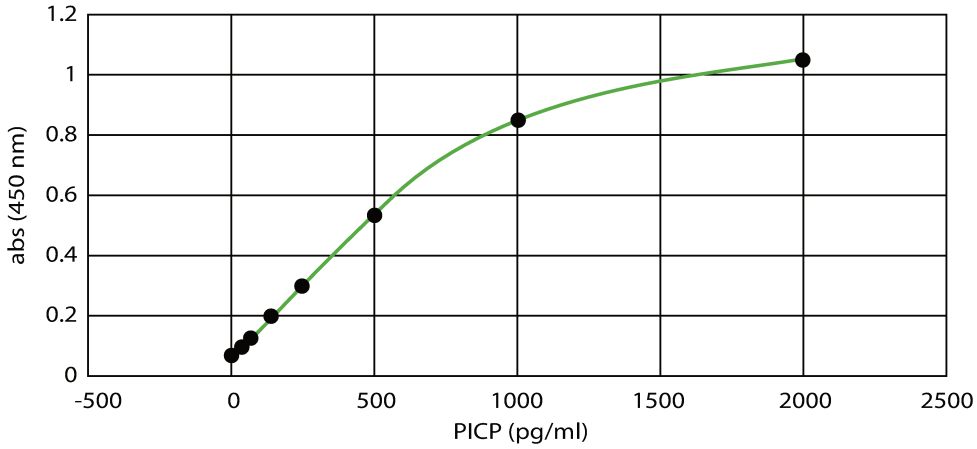

Figure 4. Sample standard curve for the Uscn ELISA Sample standard curve generated by the ELISA kit for the measurement of PICP in serum (Uscn Life Science Inc.)

Serum CrossLaps® ELISA for the quantification of degradation products of C-terminal telopeptides of Type I collagen [CTx] (Nordic Bioscience Diagnostics, Herlev, Denmark)

Fifty µL aliquots of thawed samples and manufacturer supplied standards and controls were pipetted into separate wells of a 96-well plate precoated with streptavidin. After this, 100 µL of a mixture of biotinylated antibody, peroxidase conjugated antibody, and incubation buffer (in a volumetric ratio 1:1:100, respectively) were added to each well using a multi-channel pipette. The plate was covered with a plate sealer and aluminum foil and allowed to incubate for 120 minutes on a plate shaker set to 300 rotations/minute (rpm). The contents of each well were aspirated, and the plate was manually washed with 300 µL of manufacturer provided wash diluted 1:50 in deionized water. The wash step was repeated four times before adding 100 µL of manufacturer supplied TMB substrate. The plate was covered and allowed to incubate on a plate shaker set to 300 rpm for 15 minutes. The reaction was stopped by adding 100 µL of manufacturer supplied 0.18 mol/L sulfuric acid stopping solution, and the plate was read on a microplate reader at an OD of 450nm.

Computer quantitation software (KC4™, Bio-Tek® Instruments Inc., Winooski, VT) was used to construct a standard curve by plotting the mean absorbance of the six standards against the corresponding known concentrations. A quadratic curve was generated, and the concentrations of samples were determined by interpolation.

3.4 Assay evaluation

The original study design provided for evaluation of the commercially available ELISAs for the novel purpose of quantifying PICP in feline serum. Thus, an evaluation protocol was developed, including the following constituents:

1) Samples were initially analyzed based strictly on manufacturer instructions with adjustments made as deemed necessary based on results and reported detection limits.

2) **Stability** testing of frozen sera required pooling samples to obtain sufficient volume. The pooled samples were stored in approximately 100 µL aliquots in anticipation of thawing the sample once, thus avoiding multiple freeze/thaw cycles. Because individual samples would be obtained sporadically over multiple weeks and batch analyzed, only storage at -80°C was feasible. Pooled samples were planned to consist of individual samples of sufficiently high volume after preliminary evaluation in conjunction with linearity testing. Ten different pooled samples were planned to be analyzed at time 0 and after freezing and thawing for 24 hours, 48 hours, 72 hours, 1 week, 2 weeks, 4 weeks, and 8 weeks. A sample volume calculation to determine the necessary volume for stability testing follows:

250 µL of each diluted sample (=100 µL /well X 2 for sample duplication + a small excess volume to facilitate easy pipetting) → At 1:10 dilution, necessary volume of serum is 25 µL X 8 experiments = 200 µL of serum for each of the 10 pools

Based on this calculation, samples that had >200 µL of serum remaining following preliminary experiments would be included in the pooled sample.

3) **Linearity** testing was based on the manufacturer reported working range of each assay. For example, the Uscn Life Science, Inc. ELISA was reported by the manufacturer to have a working range of 1.25-80.00 ng/mL with a minimum detectable serum concentration of PICP in the rat of less than 0.43 ng/mL. Reported linearity of the ELISA kit in that species was established using dilutions of 1:2, 1:4, 1:8, and 1:16. Therefore, we planned to evaluate 5 individual feline serum samples at these same dilutions in duplicate and to report the coefficient of variance (CV) of wells containing the same sample. Linearity was planned to be determined using serial dilutions of samples and comparing the measured OD and calculated concentrations of PICP in the samples to the dilution ratio. For example, a sample prepared at a dilution of 1:8 would be expected to produce a concentration half the concentration of the same sample prepared at a dilution of 1:4. When results differed from expected concentrations by less than 10%, the results were considered linear. If any serial dilutions produced values outside detectable limits, modifications to the dilution ratios were performed in subsequent experiments. In planning the necessary volume to carry out linearity testing, a sample calculation follows:

250 µL of each diluted sample (100 µL of sample/well X 2 for sample duplication + a small excess volume to facilitate easy pipetting). Calculated volumes are reported in Table 1.

Table 1: Sample volumes needed for planned linearity testing

concentration	µL serum	µL phosphate buffered saline
1:2*	125	125
1:4	62.5	187.5
1:8	31.25	218.75
1:16	15.625	234.375
	234.375 µL serum TOTAL	

** Although the colon symbol is generally used to denote ratios, sample dilutions in ELISA use the colon to denote a fraction (1:2 means 1 part diluted to a final total volume of 2 parts).*[137]

Based on this calculation and following preliminary experiments, samples that had >235 uL of serum remaining were retained for linearity testing.

Standards for acceptable linearity were established at a CV between duplicate wells < 10% and concentrations within the reported working range.[137]

4) **Intra-assay precision** was projected to be evaluated on 20 replicates of the following 3 samples within the same assay on the same day:

a) pool of serum from affected cats with high values on preliminary assay

b) pool of serum from normal cats with low values on preliminary assay

c) pool of samples with mid-range values on preliminary assay

At the manufacturer recommended 10-fold dilution, enough serum was estimated to be available to prepare 2000 µL of each sample (100 µL/well X 10 replicates= 1000 µL

+ 200 μL extra to facilitate easy calculation and provide a small surplus for easy pipetting) = 1200 μL = 120 μL serum + 1080 μL PBS)

Based on this calculation, following preliminary experiments, samples that had > 120 uL of serum remaining would be retained for precision testing.

5) **Inter-assay precision** was dependent on results of stability testing to ensure that product deterioration did not affect results of inter-assay comparisons completed on different days. Therefore, preliminary planning provided for analysis of these three pooled serum samples in 4 consecutive assays over 4 days to determine precision and the percentage change over time.

Precision would be calculated as follows[137]

CV% = (standard deviation/mean) X 100

Because 10 replicates would be repeated in each assay, the necessary serum volumes were calculated as follows:

[1200 μL = 120 μL serum + 1080 μL PBS] X 4= 480 μL serum

Thus, in order to execute the precision experiments outlined here, the total volume of serum necessary was determined to be:

120 + 480 = 600 μL serum **(**from each of the high, low, and mid-range pools).

6) **Recovery** experiments were planned to utilize a pooled sample consisting of 3 samples with high PICP concentrations. The plan was to use this sample to spike a separately pooled sample of sera with no detectable PICP. Standard recovery (in %) after spiking one pooled sample with known quantities of the sample with high PICP concentration would be calculated as follows:

$$\text{Recovery (\%)} = \frac{\text{obtained PICP concentration}}{\text{expected concentration}} \quad X100$$

The original study design planned for development of a normal reference interval derived from the PICP serum concentrations of the control population. Serum volume permitting, the effects of potential interfering agents, including lipemia, hemolysis and bilirubin, were planned to be evaluated by spiking samples with known concentrations of PICP with predetermined amounts of lipemic, hemolyzed, or icteric serum.

3.5 Post-mortem examination

The original study design included opportunity for post-mortem examination of enrolled shelter cats that were euthanized for reasons independent of this project. A post-mortem examination protocol was created in an effort to investigate the association between gross myocardial fibrosis and serum concentrations of both PICP and CTx.

Post-mortem examination and retrieval of the heart would be performed immediately after euthanasia using standard post-mortem dissection in a designated area.[139] Gross external and internal examinations would be performed with particular attention paid to regions of potential fibrosis. These included but were not limited to evaluation of the skin for masses, ulcerations or scars, and careful palpation of long bones for fractures or callus. We planned to examine the liver and lung lobes using standard longitudinal slices through the greatest dimension of the organs and to evaluate the kidneys by peeling the capsule and cutting longitudinally for visual inspection and

palpation. Any abnormalities would prompt documentation and collection of tissue samples < 0.5-1.0 centimeters in greatest dimension and storage in 10% formalin.

3.6 Statistical analysis

Statistical analysis software (Minitab®, Minitab Inc.; State College, PA) was used for graphical evaluation of data sets and execution of statistical tests. Descriptive data are reported as mean +/- standard deviation (SD) and range. Data sets collected for echocardiographic variables and serum concentrations of CTx were examined graphically and via the Anderson-Darling normality test and determined to fit a normal distribution. Between groups comparisons of these variables and data sets that fit a normal distribution following square-root transformation was performed using a 2 sample T-test. Data sets for variables which failed to fit a normal distribution despite log and square root transformation were compared between groups using Mann-Whitney tests. Statistical significance for all tests was defined as $P < 0.05$.

IV. RESULTS

4.1 Participants

Fifty-six cats were enrolled in the study. Nine cats were excluded from the study: 6 for fractious behavior that precluded atraumatic phlebotomy, and 3 for echocardiographic diagnoses of restrictive cardiomyopathy (n=1), ventricular septal defect (n=1), and mitral valve dysplasia (n=1). The remaining 47 cats included 28 with an echocardiographic diagnosis of HCM and 19 normal (N) controls. The mean age +/- SD of all enrolled cats was 6.98 +/- 3.73 years (HCM: 8.13 +/- 3.50, range: 2-16 years, N: 5.29 +/- 3.50, range: 1.5-15 years). Cats in the HCM group were significantly older than cats in the N group (p=0.01). There were 31 castrated males, 15 spayed females, and 1 intact female. There were 29 Domestic Shorthair cats, 11 Domestic Longhair cats, 2 Ragdolls, 2 Bengals, and 1 each of the following breeds: Rex, Maine Coon, and Russian Tabby.

Of the cats with HCM, reasons for evaluation included incidental murmur and/or arrhythmia identification, routine monitoring of previously diagnosed HCM, dyspnea, and breed screening. Four of 28 cats with HCM (=13.8%) were concurrently diagnosed with congestive heart failure at the time of presentation.

4.2 Echocardiography

Echocardiographic data are displayed in Table 2. As expected in accordance with echocardiographic diagnoses, the HCM group had significantly larger IVS and LVFW

measurements at all points when compared to the N group. The HCM group also had a larger LA:Ao than the N group measured in both 2D ($p<0.01$) and M-mode ($p<0.01$). There was no difference between the HCM and N groups for measurements of fractional shortening ($p=0.52$) nor in the diameter of the LV in either systole ($p=0.28$) or diastole ($p=0.25$). Seventeen of 28 cats (60.1%) with HCM had subendocardial hyperechogenicity, 8 with panmyocardial distribution and 9 with focal or multi-focal distribution (including papillary muscles).

Table 2: Echocardiographic measurements in cats with hypertrophic cardiomyopathy (HCM) vs. normal (N) controls

	HCM (n=28)	N (n=19)	p value
IVSd	5.12 (1.2)	4.07 (.53)	<0.01
IVSs	7.16 (1.13)	5.71 (.75)	<0.01
LVIDd	14.56 (2.78)	15.43 (2.26)	0.25
LVIDs	8.33 (.163)	9.01 (.101)	0.28
LVFWd	5.75 (1.44)	4.26 (.55)	<.01
LVFWs	7.7 (1.45)	5.89 (.67)	<.01
FS	42.57 (8.53)	40.95 (8.36)	0.52
IVSLax1	5.10 (.819)	4.15 (.496)	<.01
IVSLax2	6.2	3.9	<.01
LVFWLax	6.76 (1.04)	4.15 (.496)	<.01
IVSSax1	5.821 (.832)	3.916 (.466)	<.01
IVSSax2	5.908 (.767)	4.037 (.538)	<.01
LVFWSax	6.5 (.919)	4.095 (.617)	<.01
LA:Ao (2D)	1.4	1.2	0.0005
LA:Ao (M-mode)	1.4	1.2	0.0001

Measurements are reported as mean diameter in millimeters (standard deviation)

4.3 Serum biomarkers: PICP and CTx

Carboxy-terminal propeptide of procollagen type I (PICP) using the Microvue™ EIA kit (Quidel® Corporation, San Diego, CA):

Serum samples from 24 cats were analyzed using this assay. Serum from 12 of the 24 cats (identified as L1-L10, and L784 and L804) was obtained from the Atlantic Veterinary College Diagnostic Services Laboratory. These 12 samples were submitted to the laboratory for evaluation unrelated to this project, and the echocardiographic status of these cats was unknown. These samples were stored at the Diagnostic Services Laboratory at 4°C for <24 hours before analysis. The remaining 12 samples were from cats with HCM which had been stored at -80°C for a mean +/- SD of 44.4 +/- 18.6 days (range 2-99 days) prior to batch analysis. Thirteen of the 24 samples were analyzed at dilutions of 1:4, 1:12, and 1:24 (based on manufacturer-recommended dilutions of 1:12). The remaining 11 samples were analyzed at dilutions of 1:1 and 1:2 after results using the initial dilutions produced concentrations under or at the very low end of the standard curves. Thus, samples were prepared with higher concentrations to attempt to achieve results within detectable limits of the assay. Thirteen of 24 samples (=54.2%, 4 samples from cats with unknown echocardiographic status and 9 from cats with HCM) produced values under the standard curves at all dilutions (Table 3).

Table 3: PICP concentrations in feline serum using the MicroVue™ EIA						
ID #	Dilution	OD @ M 405 [mean of duplicate wells]	Derived concentration (in ng/ml) based on SC	Derived concentration (in ng/ml, from previous column) X dilution	CV (%) between duplicate wells	Detectable limit (OD of standards with known concentrations provided by manufacturer)
L804	1:4	0.196	<0	<0	3.1	0.198-1.268
L804	1:12	0.186	<0	<0	7.6	0.198-1.268
L804	1:24	0.211	0.1424	3.42	4.3	0.198-1.268
L784	1:4	0.228	0.635	2.5	18.6	0.198-1.268
L784	1:12	0.189	<0	<0	8.2	0.198-1.268
L784	1:24	0.191	<0	<0	9.6	0.198-1.268
L1	1:2	0.328	0.106	0.21	10.7	0.276-1.543
L1	1:1	0.395	0.123	0.12	5	0.276-1.543
L2	1:2	0.296	0.098	0.196	31.2	0.276-1.543
L2	1:1	0.303	0.099	0.099	5.1	0.276-1.543
L3	1:2	0.153	<0	<0	2.3	0.276-1.543
L3	1:1	0.152	<0	<0	1.4	0.276-1.543
L4	1:2	0.134	<0	<0	22.1	0.276-1.543
L4	1:1	0.156	<0	<0	10.4	0.276-1.543
L5	1:2	0.179	<0	<0	12.2	0.276-1.543
L5	1:1	0.208	<0	<0	7.1	0.276-1.543
L6	1:2	0.194	<0	<0	21.8	0.276-1.543
L6	1:1	0.221	<0	<0	1.3	0.276-1.543
L7	1:2	0.197	<0	<0	8.9	0.276-1.543
L7	1:1	0.199	<0	<0	13.1	0.276-1.543
L8	1:2	0.151	<0	<0	47.1	0.276-1.543
L8	1:1	0.191	<0	<0	4.4	0.276-1.543
L2	1:24	0.157	<0	<0	n/r	0.276-1.543
L2	1:12	0.15	<0	<0	n/r	0.276-1.543
L2	1:4	0.141	<0	<0	n/r	0.276-1.543
L2	1:1	0.154	<0	<0	n/r	0.276-1.543
H2	1:24	0.162	<0	<0	n/r	0.276-1.543
H2	1:12	0.154	<0	<0	n/r	0.276-1.543
H2	1:4	0.156	<0	<0	n/r	0.276-1.543
H1	1:24	0.177	<0	<0	n/r	0.276-1.543
H1	1:12	0.17	<0	<0	n/r	0.276-1.543
H1	1:4	0.157	<0	<0	n/r	0.276-1.543
H6	1:4	0.15	<0	<0	15.1	0.162-1.887

	Table 3 (continued): PICP concentrations in feline serum using the MicroVue™ EIA					
ID #	dilution	OD @ M 405 [mean of duplicate wells]	Derived concentration (in ng/ml) based on SC	Derived concentration (in ng/ml, from previous column) X dilution	CV (%) between duplicate wells	Detectable limit (OD of standards with known concentrations provided by manufacturer)
H6	1:12	0.154	<0	<0	6.4	0.162-1.887
H6	1:24	0.161	<0	<0	10.5	0.162-1.887
H3	1:4	0.16	<0	<0	1.3	0.162-1.887
H3	1:12	0.163	0.019	0.23	6.9	0.162-1.887
H3	1:24	0.142	<0	<0	2.9	0.162-1.887
H11	1:4	0.172	0.079	0.316	4.9	0.162-1.887
H11	1:12	0.159	<0	<0	9.7	0.162-1.887
H11	1:24	0.142	<0	<0	20.9	0.162-1.887
H4	1:4	0.147	<0	<0	7.7	0.162-1.887
H4	1:12	0.152	<0	<0	1.9	0.162-1.887
H4	1:24	0.15	<0	<0	6.6	0.162-1.887
H10	1:4	0.168	0.059	0.236	5.9	0.162-1.887
H10	1:12	0.153	<0	<0	6.5	0.162-1.887
H10	1:24	0.164	0.024	0.576	2.2	0.162-1.887
H7	1:24	0.112	<0	<0	11.3	0.123-1.552
H7	1:12	0.12	<0	<0	1.1	0.123-1.552
H7	1:4	0.115	<0	<0	14.2	0.123-1.552
H8	1:24	0.102	<0	<0	18	0.123-1.552
H8	1:12	0.116	<0	<0	7.3	0.123-1.552
H8	1:4	0.114	<0	<0	10.5	0.123-1.552
H9	1:24	0.109	<0	<0	16.2	0.123-1.552
H9	1:12	0.139	0.108	1.29	5.1	0.123-1.552
H9	1:4	0.125	0.018	0.072	3.4	0.123-1.552
H5	1:1	0.175	0.715	1.43	5.4	0.123-1.552
H5	1:2	0.155	0.429	0.429	5.2	0.123-1.552
H12	1:1	0.173	0.692	1.38	2.2	0.123-1.552
H12	1:2	0.161	0.522	0.522	2.4	0.123-1.552
H13	1:1	0.149	0.347	0.694	3.6	0.123-1.552
H13	1:2	0.138	0.208	0.138	5.7	0.123-1.552

Patient identification numbers (ID #) include "H" when the sample was obtained from a cat with HCM and "L" when the sample was obtained from a cat with unknown echocardiographic status.
Dilutions are expressed as volumetric ratios.
OD: optical density at which plate reader was set; SC: standard curve generated by plate reader
n/r: not reported by software

The remaining 11 samples (= 45.8%) had variable results. Two samples (H11 and L784) produced results within detectable limits at only the lowest dilutions (highest concentrations). One sample (L804) produced results within detectable limits at only the highest dilution (lowest concentration). Three samples (H3, H9, H11) had results under the detectable limit for at least one dilution that was not the most dilute preparation. In the remaining 5 samples (L1, L2, H5, H12, and H13), all dilutions produced results at the low end of the detectable limit. Measured OD values, derived concentrations, and calculated corrections for the dilution factor were not linear in any of these 5 samples. In 3 of these 5 samples (H5, H12, H13), the coefficient of variation (CV) was <10.0% between duplicate wells. In the other 2 samples, (L1 and L2), the variation between wells was higher (CV=10.7% and 31.2%, respectively) The large number of samples that fell below detectable limits and results at the low end of the detection limit that did not respect linearity indicated unreliable detection and/or quantification of feline PICP by this ELISA, and future use of this assay was abandoned.

Based on initial assay performance, stability, precision (inter- and intra-assay) testing, and recovery assessment could not be performed as originally planned.

Enzyme-linked immunosorbent assay kit for procollagen type I C-terminal propeptide (PICP) (Uscn Life Science Inc., Wuhan, Hubei [P.R. China])

Initial assay performance studies were performed using serum from enrolled HCM cats. As this assay was designed for detection of PICP in rats, fresh rat sera was obtained and used in 4 of 10 experiments and run in concert with unknown feline samples

to evaluate assay performance. Initial samples were prepared at manufacturer recommended dilutions of 1:100 and 1:200. The mean and SD of the storage time at -80° C was 68.9 +/- 51.5 days (range 14-241 days). Three samples (H3, H4, and H7) were minimally hemolyzed, while other samples had no visible hemolysis. The standard curve reported ODs ranging from 0.065-0.864 in the first experiment designated as A. Results are reported in Table 4.

Table 4: PICP concentrations in feline serum using Uscn ELISA, experiment A

ID #	Dilution	OD @ M 450 [mean of duplicate wells]	Derived concentration (in pg/ml) based on SC	Derived concentration (in pg/ml, from previous column) X dilution factor	CV (%) between duplicate wells
H1	1:100	0.08	41.19	4119	1.0
H1	1:200	0.069	10.61	2122	11.6
H2	1:100	0.104	102.51	10251	3.4
H2	1:200	0.071	12.11	2422	0
H3	1:100	0.07	8.54	854	8.1
H3	1:200	0.064	<0	<0	12.3
H4	1:100	0.073	18.79	1879	6.8
H4	1:200	0.068	7.21	1442	1.1
H6	1:100	0.08	41.19	4119	5.2
H6	1:200	0.069	10.61	2122	11.6
H7	1:100	0.067	4.71	471	5.3
H7	1:200	0.064	<0	<0	5.2
H9	1:100	0.075	23.55	2355	0.9
H9	1:200	0.059	<0	<0	2.4
H11	1:100	0.471	946	94600	16.9
H11	1:200	0.298	547.51	109502	9.0
H12	1:100	0.64	1368.2	136820	8.2
H12	1:200	0.338	636.5	127300	5.9
H15	1:100	0.083	54.249	5424.9	10.4
H15	1:200	0.068	7.21	1442	9.4
H17	1:100	0.058	<0	<0	1.2
H17	1:200	0.056	<0	<0	2.4
H20	1:100	0.07	8.54	854	7.1
H20	1:200	0.063	<0	<0	1.1
H21	1:100	0.073	18.79	1879	1.9
H21	1:200	0.071	12.11	2422	5.9
H22	1:100	0.057	<0	<0	1.3
H22	1:200	0.057	<0	<0	2.5
H23	1:100	0.068	7.21	721	4.2
H23	1:200	0.061	<0	<0	11.6
H24	1:100	0.067	4.71	471	1.2
H24	1:200	0.064	<0	<0	3.2
H25	1:100	0.062	<0	<0	8.2
H25	1:200	0.059	<0	<0	3.5
N19	1:100	0.069	10.61	1061	1.0
N19	1:200	0.06	<0	<0	11.8

Patient identification numbers (ID #) include "H" when the sample was obtained from a cat with HCM and "N" when the sample was obtained from a cat in the normal group. Samples were diluted in 0.02 molar phosphate buffered saline, and dilutions are expressed as volumetric ratios. OD: optical density at which plate reader was set; SC: standard curve generated by plate reader software

In experiment A, 3 samples (H17, H22, and H25) produced values under detectable limits at both recommended dilutions, and 7 samples (H3, H7, H9, H20, H23, H24, and N19) produced values under detectable limits at the 1:200 dilution. The remaining 8 samples (H1, H2, H4, H6, H11, H12, H15, and H21) produced values on the standard curve at both dilutions but the calculated concentrations were not linear. In 4 of those 8 samples, the CV between duplicate wells was acceptably low (<10%), making operator error an unlikely source for the lack of linearity. In the remaining 4 samples, the CV values were 10.4%, 11.6%, 11.6%, and 16.98%, respectively, making operator error a potential source for the lack of linearity.

In the next experiment designated as B, samples from fewer cats were analyzed at dilutions of 1:10, 1:20, 1:50, 1:100, and 1:200. These dilutions were selected because some samples in experiment A failed to produce values within detectable limits at the most dilute preparation (1:200). Therefore, samples in experiment B were prepared at higher concentrations in an attempt to produce results within detectable limits of the assay as determined by the standard curve (Figure 5). Results are reported in Table 5.

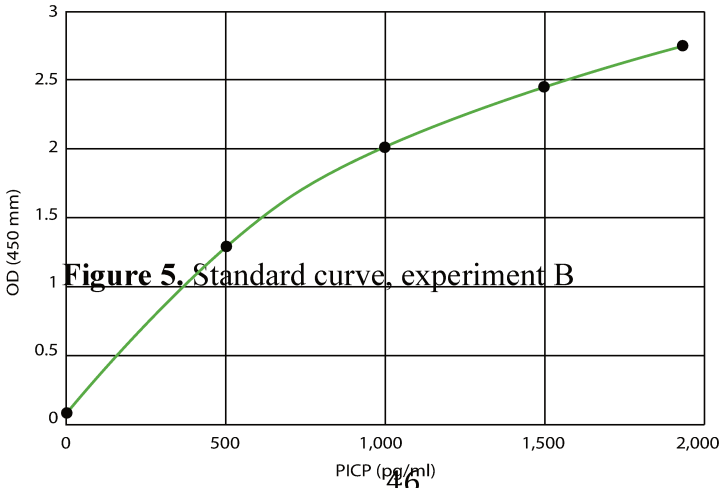

Figure 5. Standard curve, experiment B

Table 5: PICP concentrations in feline serum using Uscn ELISA, experiment B

ID #	Dilution	OD @ M 450 [mean of duplicate wells]	Derived concentration (in pg/ml) based on SC	Derived concentration (in pg/ml, from previous column) X dilution	CV (%) between duplicate wells
H1	1:10	3.498	>2000	>20,000	n/r
	1:20	2.876	>2000	>40,000	n/r
	1:50	1.289	531.01	26,550.5	8.4
	1:100	0.735	252.76	25,276.0	6.5
	1:200	0.401	123.32	24,664.0	0.2
H2	1:10	1.654	752.17	7,521.7	9.8
	1:20	1.414	603.23	12,064.6	6.7
	1:50	0.667	222.83	11,141.5	9.3
	1:100	0.361	110.98	11,098.0	0.9
	1:200	0.222	61.278	12,255.6	3.4
Rat	1:10	1.494	658.42	6,584.2	30.9
	1:20	1.077	416.96	8,339.2	1.0
	1:50	0.593	191.84	9,592.0	10.8
	1:100	0.319	98.15	9,815.0	10.7
	1:200	0.197	46.09	9,218.0	6.7
H4	1:10	.517	163.0	1,630.0	12.6
	1:20	.265	79.3	1,586.0	12.9
	1:50	.135	17.04	852.0	17.5
	1:100	.112	9.08	908.0	41.8
	1:200	.095	3.28	656.0	35.4

Patient identification numbers (ID #) include "H" when the sample was obtained from a cat with HCM and "N" when the sample was obtained from a cat in the normal group.
Samples were diluted in 0.02 molar phosphate buffered saline and are expressed as volumetric ratios. OD: optical density at which plate reader was set; SC: standard curve generated by plate reader software. CV: coefficient of variation

In

experiment B, three previously evaluated samples and one fresh sample of rat serum all produced results on the associated standard curve but the results failed to demonstrate appropriate linearity. In at least two cases (H1 and H2), this occurred despite acceptably low variation. One sample (H4) with non-linear results had high (12.6-41.8%) CVs between duplicate wells.

Based on the imperfect linearity in the rat sample despite reasonable variation, the experiment was repeated using a different sample diluent. Phosphate buffer saline was replaced with serum from a sample that did not produce a measurable concentration of PICP. In this experiment (data not shown), samples H1, H2, and H4 were all prepared in the same dilutions as in experiment B and serum sample H17 (which previously produced no detectable PICP at 1:100 and 1:200 dilutions with CV values <3%) was used as the diluent. The results produced an acceptable standard curve, but the unknown samples produced OD values below detectable limits. The rat sample was not included in this experiment due to inadequate volume.

Samples analyzed in both experiments A and B included H1, H2, and H4. In experiment A, these samples produced values within detectable limits of the assay but failed to do so repeatedly in experiment B, suggesting poor inter-assay precision. Closer evaluation of precision was performed with serial evaluation of a separately pooled sample at different time points. The pooled sample contained serum from 3 HCM cats (H7, H8, and H9) whose serum samples were collected and centrifuged within 8 hours, which allowed for serum mixing before the first freeze-thaw cycle. Results of preliminary precision testing are summarized in Table 6.

Table 6: Precision evaluation of Uscn ELISA using one pooled feline serum sample

Time of evaluation	OD @ M 450 [mean of duplicate wells]	Derived conc (from accompanying standard curve)	CV (%) between duplicate wells
0	0.343	2854	12.6
24 hours	0.162	429.4	11.2
48 hours	0.156	74.5	13.7
2 weeks	0.014	<0	n/r

OD: optical density at which plate reader was set; CV: coefficient of variation.
n/r: not reported. CV was not reported by the software when the derived concentration was 0.

The CV between times 0 and 24 hours was 104.4% and between 24 and 48 hours was 99.6%. The variation at two weeks could not be determined as the OD was below detectable limits.In an effort to address potential of operator error as a cause for variability, additional performance testing was undertaken by an independent and experienced laboratory technician using the same samples and ELISA kit. Linearity and repeatability parameters were again unacceptable (data not shown) despite acceptably low variation between duplicate wells. Remaining experiments were conducted under the supervision of this experienced laboratory technician proficient in ELISA work. Samples were routinely analyzed at various dilutions with and without concurrent evaluation of fresh serum samples from rats. Preferential analysis of samples producing non-linear results within detectable limits in experiments A and B was performed. The standard curve (OD detectable limits: .084-1.031, Figure 6) and results (Table 7) follow.

Figure 6. Standard curve, experiment C

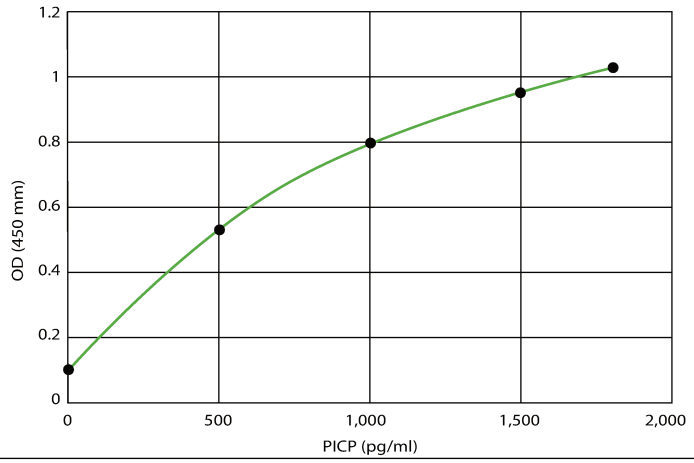

Table 7: PICP concentrations in cat serum using Uscn ELISA, experiment C

ID #	Dilution	OD @ M 450 [mean of duplicate wells]	CV (%) between duplicate wells	Derived concentration (in pg/ml) based on SC	Derived concentration (in pg/ml, from previous column) X dilution	CV (%) between calculated concentrations
RAT1	1:50	0.386	1.89	589.07	29453.5	
RAT1	1:100	0.301	2.32	280.1	28010	2.65
RAT1	1:200	0.214	1.98	141.7	28340	
H12	1:50	0.07	n/r	<0	<0	
H12	1:100	0.065	n/r	<0	<0	n/r
H12	1:200	0.066	n/r	<0	<0	
H15	1:50	0.086	n/r	2.2	110	
H15	1:100	0.073	n/r	<0	<0	n/r
H15	1:200	0.071	n/r	<0	<0	
H21	1:50	0.075	n/r	<0	<0	
H21	1:100	0.059	n/r	<0	<0	n/r
H21	1:200	0.078	n/r	<0	<0	
H23	1:50	0.072	n/r	<0	<0	
H23	1:100	0.08	n/r	<0	<0	n/r
H23	1:200	0.068	n/r	<0	<0	
H24	1:50	0.117	7.89	43.1	2155	
H24	1:100	0.112	9.07	40.7	4070	43.5
H24	1:200	0.062	n/r	<0	<0	
H25	1:50	0.075	n/r	<0	<0	n/r
H25	1:100	0.076	n/r	<0	<0	n/r
H25	1:200	0.067	n/r	<0	<0	n/r
H26	1:50	0.437	10.47	397.1	19855	
H26	1:100	0.167	2.32	124.5	12450	29.6
H26	1:200	0.128	2.23	60.46	12092	

Patient identification numbers (ID #) include "H" when the sample was obtained from a cat with HCM .
Dilutions are expressed as volumetric ratios. OD: optical density at which plate reader was set; SC: standard curve generated by plate reader software. CV: coefficient of variation. n/r: not reported.

In experiment C, the rat sample diluted 1:100 and 1:200 produced concentrations that were linear with acceptably low CV values. The derived concentration of the 1:50 preparation of the rat sample was less than twice the derived concentration of the 1:100 preparation. The other samples produced either nonsensical (H24) results or results under the detectable limit of the assay (H12, H15, H21, H23, and H25) with the exception of one sample (H26). This sample resulted in derived concentrations that were linear at 1:100 and 1:200 dilutions. The derived concentration of the 1:50 preparation of this sample was greater than twice the derived concentration of the 1:100 preparation. This sample (H26) was re-analyzed in another experiment on the following day along with 2 others from HCM cats, and results are reported in Table 8:

Table 8: PICP concentrations in feline serum using Uscn ELISA, experiment D

ID #	Dilution	OD @ M 450 [mean of duplicate wells]	CV (%) between duplicate wells	Derived concentration (in pg/ml) based on SC	Derived concentration (in pg/ml, from previous column) X dilution	CV (%) between calculated concentrations	Detectable limit (OD of standards with known concentrations provided by manufacturer)
RAT2	1:50	0.423	5.41	1156.2	57810	0.76	.050-.424
RAT2	1:100	0.209	8.5	571.12	57112		.050-.424
RAT2	1:200	0.116	10.78	285.05	57010		.050-.424
H9	1:50	0.068	30.16	94.1	4705	41.3	.050-.424
H9	1:100	0.061	0	74	7400		.050-.424
H9	1:200	0.053	1.37	55.3	11060		.050-.424
H13	1:50	0.058	14.7	62.94	3147	n/r	.050-.424
H13	1:100	0.048	n/r	<0	<0		.050-.424
H13	1:200	0.044	n/r	<0	<0		.050-.424
H26	1:50	0.085	3.04	171.52	8576	23.09	.050-.424
H26	1:100	0.068	3.57	102.15	10215		.050-.424
H26	1:200	0.045	0	67.27	13454		.050-.424

Patient identification numbers (ID #) include "H" when the sample was obtained from a cat with HCM . Dilutions are expressed as volumetric ratios. OD: optical density at which plate reader was set; SC: standard curve generated by plate reader software. CV: coefficient of variation. n/r: not reported.

Results of experiment D, like the previous one, supported appropriate performance of the ELISA using rat serum since serial dilutions in the recommended preparations produced linear results with low CV values between calculated concentrations. The H26 feline sample failed to produce linear results despite low CV values between duplicate wells. In addition, the calculated concentrations over a 24 hour time period varied from 8576 to 19855 pg/ml, suggesting poor stability or poor precision.

The results of initial assay performance evaluation suggested that the assay did not reliably detect or quantify PICP in feline serum. Based on the repeated lack of linearity and repeated lack of precision, the use of this assay for determining PICP concentration in cat serum was discontinued. As such, stability, precision (inter- and intra-assay) testing, and recovery assessment could not be performed as originally planned.

Serum CrossLaps® ELISA for the quantification of degradation products of C-terminal telopeptides of Type I collagen (immunodiagnostic systems, Herlev, Denmark)

All serum samples were batch analyzed over the course of 4 separate experiments run on 4 different days. The mean sample storage time in -80°C was 89.8 days (range 7-201 days). The mean +/- SD serum [CTx] in the HCM group was 0.248 +/- 0.224 ng/ml. The mean +/- SD serum [CTx] in the control group was 0.253 +/-0.225 ng/ml. There was no difference in serum [CTx] between the groups (p=0.4).

4.4 Post-mortem examination

Based on the inability of either PICP assay to detect or quantify PICP in feline serum, no post-mortem examinations were performed. There are, therefore, no results regarding histologic identification of myocardial fibrosis to report.

V. DISCUSSION

5.1. Overview of objectives

The primary goal of this project was to evaluate serum biomarkers of collagen turnover in cats with HCM as surrogate markers for myocardial fibrosis, a distinct pathologic manifestation of HCM. Actions required to address this goal included assessing two commercially available assays for quantifying PICP in feline serum, comparing serum concentrations between cats with HCM and normal cats, and determining whether or not an association existed between biomarker concentrations and the presence or extent of myocardial fibrosis. At best, a difference in these concentrations between normal and affected cats would provide insight into the pathophysiology of HCM. In addition, serum biomarkers could provide an alternate form of disease recognition, perhaps even prior to the onset of echocardiographic changes, as was recently shown in people.[24] At worst, no detectable difference would refute the diagnostic yield of identifying myocardial fibrosis using concentrations of these serum biomarkers. One major challenge at the onset of the project was the lack of a gold standard test for identifying myocardial fibrosis, much less surrogate markers for myocardial fibrosis, in the cat.

5.2 Myocardial fibrosis

Myocardial fibrosis is not specific to the pathophysiologic progression of HCM but is commonly recognized as a result of hypertensive heart disease and myocardial infarction in humans..[13,21,118,120] The interest in recognizing myocardial fibrosis in human

HCM patients revolves around recent evidence that supports an association between myocardial fibrosis and progressive congestive heart failure, increased arrhythmias, and sudden cardiac death.[111,113,119] Fibrotic tissue has poorer electrical conductance than normal myocardial conductive tissue, which interrupts normal impulse transmission and enables electrical reentrant pathways, thus creating substrates for ectopy.[75,77,88,89] These mechanisms of arrhythmogenesis have previously been shown to result in poor clinical outcomes in humans with HCM.[88-90] The clinical identification of myocardial fibrosis and mitigation of arrhythmic complications remains practically difficult in cats.

Echocardiographic myocardial hyperechogenicity and late-gadolinium enhancement on cMRI appear to be specific but not particularly sensitive means of identifying myocardial fibrosis.[58,59] Thus, even though only 17/28 cats with HCM in this study had echocardiographic evidence of subendocardial hyperechogenicity consistent with myocardial fibrosis, it would be reasonable to suspect that the 11 cats without this echocardiographic lesion still may have histologically demonstrable myocardial fibrosis. Furthermore, it would be reasonable to expect that some cats with HCM without echocardiographic hyperechogenicity might also have elevated serum concentrations of PICP consistent with myocardial fibrosis. However, none of the cats in this study had reliably measurable serum PICP. The remainder of this discussion will focus on potential reasons why the lack of detection of PICP on two ELISAs was the major finding in this study.

5.3 Detecting PICP in feline serum: summary of why results demonstrated assay failure

During initial evaluation of assay performance using the Microvue™ EIA kit, a large number of samples fell below detectable limits. Samples that produced results within detectable limits were always at the low end of those limits. This occurred despite intentionally preparing samples at low dilutions in an attempt to concentrate any measurable PICP. Furthermore, results within detection limits did not produce linear results during serial dilution. These factors led to the conclusion that the Microvue™ EIA kit was unreliable in the detection and/or quantification of feline PICP.

These results also prompted use of a different assay, the PICP ELISA by Uscn Life Science Inc., which was used in 10 experiments. The variable results achieved using this assay warrant consideration. Generally, CVs equal to or less than 15% for raw OD values indicate adequate repeatability.[137] In all experiments using this ELISA, there were intermittent instances where the CVs for OD values between duplicate wells exceeded 15%. In those instances, operator controlled error could not be excluded as a cause for variable results. However, as the experiments continued and operator technique improved, more samples produced OD values with a CV < 15% between duplicate wells. This suggested that earlier operator controlled error had resolved and could not wholly explain the persistent variable results. When the CV between duplicate wells was less than 15%, non-linear concentrations of serial dilutions indicated inherent variability within the assay for detecting or quantifying PICP in feline serum.

Evaluation of fresh rat sera during several experiments revealed linear results within the reported working range of the assay. In experiments B, C, and D, results were

linear at dilutions of 1:100 and 1:200, the manufacturer recommended dilutions. That is, the calculated concentration of a sample prepared at 1:100 was twice that of the same sample prepared at 1:200. Linear results were routinely not maintained when more concentrated samples were prepared and analyzed. We suspected that at higher concentrations, non-specific protein binding erroneously produced higher OD values. Demonstration of linearity at manufacturer recommended dilutions suggested that the assay did not exhibit inherent variability when measuring PICP in rat serum, a use for which the assay is labeled. Therefore, we concluded that despite proper assay performance, detection or quantification of PICP in feline serum was not reliable.

Potential explanations regarding the unreliable results generated from the 2 commercially available ELISAs for PICP include the following:

1) Operator error in the execution of experiments
2) Lack of PICP in the samples
3) Lack of cross reaction between the polyclonal antibody reagents in the assay and PICP in feline sera

The opportunities for operator controlled error must be thoroughly evaluated first as these errors, once recognized, are avoidable in future experiments. The investigators who developed the first radioimmunoassay for PICP detection wrote that "procollagens are difficult to handle, which easily leads to very low yields of the purified propeptide."[15] This statement suggests that meticulous technique is important in preserving the integrity of the procollagens for proper measurement. Experiments involving the Uscn ELISA routinely produced standard curves, control, and sample results within and between assays with high variability. The manufacturer provided kit insert advised that

inconsistent OD values on standard curves were expected when conditions of assay performance varied. These conditions included pipetting and washing techniques and temperature and storage effects of the kits and samples.

An exhaustive review of operator controlled sources of variability is warranted. First, the variation could not be explained by product expiration as all kits were used before their expiration dates and all reagents were stored as specified by manufacturers. Before each experiment, the reagents were visually inspected for precipitation, discoloration or other visual signs of instability or deterioration. No such signs were identified. On one occasion, a newly opened kit had an empty vial of the most concentrated (2.000 pg/ml) standard. A company representative recommended use of remaining standard from a previously used kit, acknowledging the potential for variability given the separate kit lot numbers. Based on visual inspection of the resulting standard curve (Figure 7), the appearance of sample concentrations at the low end of that curve, and the low CV between duplicate wells, the standard from the previous kit was considered acceptable.

All reagents and samples were gently mixed before addition to the wells, and caution was taken to avoid foaming during mixing as foaming can denature proteins.[137] All manufacturer recommendations for incubation times and temperatures were strictly adhered to and recorded. During the washing process, one modification to the recommended protocol was performed as described previously. The manufacturer recommended adding 350 µL of prepared wash solution to each well during each of the 5 washing steps. During the initial experiment, it was noted that this volume exceeded the capacity of the well and formed a bubble at the top of the well. Because this much

volume had considerable potential to contribute to spilling and inter-well contamination, the volume of wash solution was reduced from 350 μL to 300 μL in the following experiments. Caution was taken to carefully aspirate all fluid from the wells during each wash and to avoid letting the wells sit dry. Pipette tips were routinely visually inspected during multi-channel pipetting for the aspiration of uniform amounts of reagent among the tips and for the presence of air bubbles. Air bubbles and/or discrepancies in pipette volumes prompted ejection of the fluid from the pipette and changing pipette tips. In addition to the guidance and supervision of experienced laboratory technical staff, these measures to ensure proper sample handling, pipetting, and washing techniques were believed to reduce operator error as much as reasonably possible. Based on a general trend of improvement in variation as the experiments progressed, operator error is unlikely to explain the unreliability of both assays in the quantification of PICP in feline serum.

A second explanation for unreliable PICP detection and/or quantification is the lack of PICP in samples. This may have occurred because cats included in the study had no PICP in serum or because the PICP denatured during storage prior to analysis. The hypothesis that serum samples from cats included in this study contained no PICP is unlikely. A major constituent of the bony matrix and all connective tissue, type I collagen is among the most abundant proteins in the animal body, and even a cat without bone disease or excessive fibrosis would be expected to have a measurable amount of PICP secondary to normal bone turnover.[138] Just as there are recognized reference intervals for male and female humans at various ages, so too would one expect to be able to measure this protein in normal feline sera.[16,101,129,138,140]

Without reliable stability studies, it is impossible to refute the possibility that measurable amounts of PICP were originally present in serum samples and subsequently degraded during storage. Proper sample handling was demonstrated by atraumatic phlebotomy, rapid transfer to collection tubes, vigilant monitoring for clotting followed promptly by centrifugation and serum transfer to an -80°C freezer. Given the friable nature of procollagens and the unknown detection capacity of the assays in use, these principles of sample handling were adhered to from the first enrolled study participant. Alternative forms of sample harvesting, particularly use of a proteinase inhibitor in the storage tube or use of a chilled centrifuge, may be indicated in future investigative attempts to quantify PICP in feline sera. Although neither of the ELISA kit inserts recommended such measures, they may be necessary if PICP is less stable in feline serum in long-term storage compared to humans. Procollagen C-proteinase (also called bone morphogenetic protein-1) is the endoproteinase that cleaves PICP off the procollagen precursor molecule.[141] Its inhibition has become an attractive target for anti-fibrotic therapy since disruption of propeptide cleavage would theoretically prevent collagen from trimerizing into functional fibrils.[141] Despite research interest in developing an inhibitor to procollagen C-proteinase, no proteinase for use *in vitro* has been established for preserving PICP in serum during storage.

It is a limitation of this study that batch analysis resulted in longer than desired storage time. The original study design acknowledged this limitation and provided for immediate storage of all samples at -80°C. This precluded evaluation of storage at room temperature, 4°C, and -20°C. Mean storage times exceeded one of the PICP kit manufacturers (Quidel® MicroVue™) published recommendations (<2 months), while

manufacturers of the other ELISA kit did not specify recommendations regarding storage time. Stability data was not published in previous investigation of the Serum CrossLaps® ELISA for CTx, but the primary investigator reported that serum samples were stored at -80°C for well beyond six months (personal communication).[138] Although our mean storage time fell below this reported storage time, proper stability evaluation is necessary to determine the effect of storage on PICP in feline serum.

In assay evaluations and certainly when pursuing assay validation, assessing assay performance on samples stored under varying conditions is essential. In this project, stability testing of frozen samples was only performed at times 0, 24 hours, 48 hours, and 2 weeks. Ideally, more than one sample would be evaluated at more frequent intervals over a greater period of time. The unreliable detection of PICP and the temporal variation in one pooled sample precluded further stability testing.

Perhaps the most likely explanation for the unreliable detection of PICP in this project is incompatibility between PICP in feline serum and the two antibodies provided by the manufacturers of the assay kits. Without the known protein sequence of PICP in cats, it is impossible to comment on its degree of homology to the human or rat, respectively, proteins that these two kits were designed to detect. Purification and protein sequencing of feline PICP would ideally enable investigators to determine if cross-reactivity with other species is expected. If not, novel assay development could be performed to detect PICP in feline serum. In doing so, antibody-antigen binding could be detected using a biosensor, and an immunoassay using surface plasmon resonance (SPR) could detect a change in mass on the surface of an antibody-coated sensor chip, which occurs in antigen binding.[141] The binding component could then be identified by

purification and protein sequencing. It was this technique that enabled investigators to purify guinea pig PICP and develop a competitive ELISA for its detection and quantification.[141] Time permitting, this would have been the appropriate course of action to address the unreliable detection of PICP in feline serum in this study and is recommended as a step for future research.

5.4 Collagen degradation

The finding that serum CTx concentrations did not differ between HCM and normal cats was not unexpected. In Ho et al.'s evaluation of collagen biomarkers in human probands with HCM, there was no significant difference in the marker of collagen degradation (ICTP) between mutation carriers with and without phenotypic expression of disease.[24] In that study, the PICP:ICTP ratio, a reflection of the balance between collagen synthesis and degradation, was not different between normal and mutation-positive, LVH-negative groups but was significantly higher in subjects with overt HCM. Investigators surmised that these biomarker alterations implicated excessive collagen synthesis without degradation in HCM.[24] Thus, despite the inability to demonstrate elevated serum concentrations of PICP in cats with HCM in this study, the CTx results support no difference in collagen degradation between cats with HCM and normal controls. Cats with myocardial fibrosis would be expected to have increased PICP without concordant increases in CTx.

Since CTx was previously quantified in feline serum, our results were compared to published reference intervals.[129] In healthy cats ranging from 1-10 years of age,

DeLaurier et al. reported that reference intervals (mean +/- 2 standard deviations) of CTx varied between age groups (age 1-2 years: 6194 +/- 3929 pg/ml; age 3-5 years: 3290 +/- 1744 pg/ml; and age 6-10 years: 3228 +/- 1891 pg/ml).[129] The mean serum CTx concentrations from HCM and N cats in the present study (HCM: 248 pg/ml, controls: 253 pg/ml) were approximately 10 times lower than mean concentrations in the previously published study[129] for unknown reasons. In a separate study evaluating serum biomarkers of bone turnover, healthy, geriatric cats were reported to have a median serum CTx concentration of 336 pg/mL (range 231–642 pg/ml).[138] The CTx concentrations measured in our HCM and N groups are more similar to the CTx concentrations of geriatric cats (age not specified) in this study than in the larger and variably aged population reported by DeLaurier et al.[129,138]

5.5 Post-mortem evaluation of myocardial fibrosis

Deviation from the original project design occurred because of the unreliability of assay results. No post-mortem examinations were pursued or performed because there were no cats enrolled from the Prince Edward Island Humane Society after initial assay evaluation. Ideally, histologic evaluation for the presence and extent of myocardial fibrosis would be compared to serum levels of biomarker concentrations. This relationship and any deviations in cats with HCM over normal cats could provide a means for evaluating the presence of myocardial fibrosis in cats.

VI. CONCLUSIONS

In this project, the negative results of assay performance for quantifying PICP in feline serum has important consequences. Though the potential implications are clinical, research needs to start in the laboratory with proper isolation and purification of PICP in feline serum since results of the present series of experiments suggest that it eludes detection on human and rat specific assays. The evaluation of myocardial fibrosis in cats remains a worthwhile pursuit, particularly as evidence mounts that the presence and extent of myocardial fibrosis is a negative prognostic indicator in people with HCM.

As expected, cats with HCM have significant differences on echocardiographic evaluation compared to normal cats but do not have significantly different median serum concentrations of CTx, a marker of collagen degradation. This latter conclusion would have been an important consideration in conjunction with PICP concentrations in both groups, had we successfully quantified them.

Perhaps the addition of histologic evaluation of the myocardium would provide more sensitive detection or more direct evidence of the difference in the ECM of cats with and without HCM. In addition to detecting and measuring PICP in cat serum, future research would optimally be aimed at comparing serum concentrations of collagen biomarkers to histologic evidence of myocardial fibrosis.

If serum biomarkers of collagen turnover do not implicate myocardial fibrosis in the pathogenesis of HCM, then alternate roles of this histologic manifestation of disease should be considered. It is currently understood that HCM arises from genetic mutations encoding sarcomeric proteins, altering their function and causing myocyte hypertrophy in order to maintain force generation.[27] Myocardial fibrosis is not considered causal in the

development of HCM. Instead, it occurs as a result of myocyte ischemia secondary to underperfusion of the hypertrophied cells. Ho et al.'s work is important because, for the first time, it suggests that myocardial fibrosis may not be solely a consequence of disease, but may also contribute to early pathogenesis.[24] Myocardial fibrosis may even propagate hypertrophy by further impeding myocyte force generation. The association between fibrosis and arteriosclerosis is also intriguing, and one might wonder whether the fibrotic myocardium induces vascular changes. Specifically, it might be suggested that the dysfunctional fibrotic myocardium increases oxygen demand that spurs vascular remodeling, promoting further reparative fibrosis.

The need for continued effort in the investigation of myocardial fibrosis is evident. In human patients with HCM, serum collagen biomarkers have provided a means for early disease detection. Yet the assays remain largely limited to research purposes without wide clinical availability. Furthermore, whether these biomarkers change with disease progression or influence treatment or prognosis remains unknown. Long-term observational longitudinal studies of both symptomatic and asymptomatic individuals could address these questions.

The quest for investigating myocardial fibrosis in cats with HCM is broader. Not only have we failed to identify measurable changes in serum collagen biomarkers in cats, but imaging modalities that routinely identify fibrosis in human hearts are less divulging in cat hearts. We might hypothesize that the development of myocardial fibrosis in HCM occurs differently in cats than in humans. Perhaps species differences limit the degree of fibrosis to only that which occurs as a reparative process in cats. Perhaps the difference in lifestyles between humans and sedentary domestic cats limits the degree of myocardial

stress in cats with HCM. Therefore, maybe there is less cellular impetus for myofibroblasts to secrete excessive collagen in the disease state. All of these questions provide avenues for future research.

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